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AN EPIDEMIC DISEASE AFFECTING SALMON AND TROUT IN ENGLAND DURING THE SUMMER OF 1911[1].

By JOSEPH A. ARKWRIGHT, M.D.

(*From the Lister Institute, London.*)

Epizootics of various Fish-diseases due to bacteria have been described by several observers, but most of these have affected coarse fish only, such as carp, tench and perch and the bacilli, which have been described as the causative organisms, have been quite different in their characters from those constantly found in the epizootic now under consideration.

Patterson (1898) described *Bacillus salmonis pestis* and showed that this bacillus was the essential microorganism present in "Salmon-disease," an infective disease occurring in the late Autumn and Winter months. Epidemics of the disease known as 'Furunculosis of the Salmonidae' have been observed in many parts of Europe, but the occurrence of this disease in the British Isles has not been hitherto recorded.

Previous observations by other writers.

Emmerich and Weibel (1894) published a detailed account of Furunculosis of the Salmonidae and described *Bacillus salmonicida* as the cause of the disease. They recorded an epidemic occurring in the Autumn (October) of 1888 in the tanks of a fish culture establishment. *Trutta fario* (trout) and *Salmo fontinalis* were attacked, but Rainbow-trout escaped. The disease was characterised by congestion of the intestine, especially the mid and hind gut, and in many instances by haemorrhages in the muscles followed by patches of softening and ulceration with discharge of "pus" through an opening in the skin. Discharging sinuses often remained for some weeks. The affected fish usually died after one or two weeks, but occasionally recovered. *B. salmonicida* was isolated, often in pure culture, from the blood, the muscular haemorrhages and internal organs. This organism they described as a small non-motile Gram-negative bacillus which grew slowly on artificial media at the temperature of the laboratory. The optimum temperature for growth was between 10° C. and 15° C., but slight growth occurred almost down to freezing point. No growth occurred at 37° C. and the bacilli were rapidly killed at 60° C.

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[1] This paper originally appeared as a Report (Arkwright, 1912) to the Board of Agriculture and Fisheries by whose permission it is now republished in a slightly altered form.

On gelatin the growth was slow and only very small colonies appeared during the first two or three days. Later, liquefaction of the gelatin with gas production occurred. They considered the appearance of stab gelatin cultures as characteristic. The growth on agar was whitish-grey at first and later became yellowish. After some weeks however the growth became brownish and the upper part of the agar in the culture tube also became brown. They claimed to have shown that the disease could be communicated to healthy fish by artificially contaminating the water of tanks with pure cultures. Marsh (1903) in the United States of America described an organism—*Bacterium truttae*—found by him in fish which had died of an epizootic disease occurring amongst trout in captivity. He does not mention the symptoms of the disease nor the pathological changes found. His description of *B. truttae* agrees very well with the characters of the bacillus isolated from fish which died during the English epidemic in 1911, and in particular, he mentioned the production of a diffusing brown pigment in the cultures.

Hofer (1904) in describing Furunculosis of the Salmonidae stated that he had repeatedly confirmed Emmerich and Weibel's observations on the disease and on *B. salmonicida*. He says that infected fish are not harmful when used as human food. Plehn (1909) in a short note stated that the *B. salmonicida* isolated from diseased fish examined during a recent epidemic corresponded to the original description of the bacillus, but also mentioned that the gelatin in cultures became slightly brown in ten days and "coffee-brown" in three weeks, also that broth cultures developed a brown colour though rather more slowly. On agar her strain made very scanty growth and showed involution forms; no mention is made of pigment formation. Plehn (1911) described more fully the epizootic of 1909. Between 1894, the date of Emmerich and Weibel's paper, and 1909, fish from different parts of Germany which had died from Furunculosis were yearly examined at Munich and were found to be infected with *B. salmonicida*. These diseased fish were Trout and "Bach-saibling" but never Rainbow-trout. All the diseased fish were reported from fish-culture establishments and none from open water.

In 1909 and 1910 large numbers of fish died and many diseased fish were obtained from open streams and rivers in widely separated parts of Germany. Twenty-five rivers and streams were affected in Bavaria alone in 1909. Many grayling were attacked and a salmon from the Rhine was found to be suffering from this disease. Some tench in a tank were infected by diseased trout living in the same water, and a pike from an open stream was found to be infected. The haemorrhages and "abscesses" appeared to be less common than in former epidemics, and many of the fish had no lesions except congestion of the intestine. The tench had no macroscopic appearances of disease but the pike showed ulcers and abscesses. "Furunculosis" was also reported from Switzerland and France. The strains of *B. salmonicida* isolated by Plehn during these two years showed some characters on culture which differed from those of the bacillus isolated in former epidemics. Plehn calls this new strain *B. salmonicida-B.* The stab cultures in gelatin of this recent strain were less characteristic and the track of air bubbles along the line of liquefaction was less marked and less constant. A very characteristic brown colour appeared in the gelatin and broth cultures after the second week. This pigment was most marked near the surface of the culture. Pigment formation on agar was later and much less pronounced. A very slight acid reaction of the medium was found to inhibit growth markedly and an even smaller amount of acidity prevented the formation of pigment.

The bacteria were killed at 40° C. and growth was very much weakened at 30° C.

Trout were experimentally infected by feeding and by adding broth culture to the water, but all trout exposed to infection in this way did not succumb. Some yearling trout which had escaped infection by feeding were, six weeks later, put with healthy trout. Some of these latter became ill and died, and were found to be infected. The healthy trout which had introduced the infection were then killed and were also found to be infected. One trout which was exposed to infection by water containing culture, became ill and recovered. The water in which it was kept was then raised in temperature from 8° C. to 15° C. with the result that this fish developed septicaemia with *B. salmonicida* and died. These latter experiments, if they were sufficiently controlled, show that apparently healthy trout can be carriers of *B. salmonicida* and can infect healthy fish.

The English Epidemic during 1911.

For the following Epidemiological facts I am indebted to Dr A. T. Masterman's report to the Board of Agriculture and Fisheries (1912). During the summer of 1911 there was a high mortality amongst fresh water fish in England. The Exe, the Dart, the Teign and the Wye were principally affected, and in these four rivers in the South-west, large numbers of Salmon and Trout died between the 12th of May and the end of July. Dead fish were not reported from the Exe after the 10th of July. The summer was unusually hot and dry and the general improvement at the end of July roughly coincided with some storms of rain and a temporary diminution of the drought, at any rate in some areas. During the epizootic large numbers of fish were found dead. In the Exe before the 16th of June 225 salmon and 718 trout were reported and during three days in June 521 dead trout were taken out of the river. Over 200 dead salmon were reported from the Wye between the 25th day of May and the 30th of July. The numbers must have been in reality larger as no doubt many dead fish were never reported. In the Wye besides salmon and trout, many grayling, eels, pike, chub and dace were found dead. For a more detailed account of the Epidemic the reader is referred to Dr Masterman's report.

Examination of dead fish.

The diseased fish, which were examined, were sent to the Lister Institute through the Board of Agriculture and Fisheries. They were received during the months of May to September, 1911, but the greater number came in during June. They were taken out of four rivers or their tributaries, the Exe, the Wye, the Teign and the Dart. The diseased fish were removed from the river either dead or in a dying condition, and were sent to London in a box or basket without ice, except in the case of six fish (Trout 2 to 7) which were packed in ice. The fish were generally received on the day after they were despatched, and usually, but not always, appeared to be fairly fresh externally. The liver and spleen were, however, frequently diffluent in the case of diseased fish.

Besides the diseased fish which were sent because they were found dead or dying in the course of the epidemic, fish were also examined from other sources and served as controls.

In all, 50 fish were examined. Of these 14 were salmon[1], 34 were trout, one was a grayling, and one a bream. Thirteen of the salmon were sent because they were considered to be suffering from the epidemic disease, whilst the remaining salmon was caught alive in a net in the Wye and was not obviously diseased though it had lost some scales.

Of the 34 trout examined *post-mortem* 11 had been kept alive in the laboratory for varying lengths of time and attempts had been made to infect most of them. These are called "experimental trout" in the tables.

Of the remaining 23 trout, seven were taken dead or dying from the Exe and were supposed to be affected by the epidemic disease. Seven were sent from a breeding-station, having died in the midst of apparent health immediately after transference from one tank to another. Eight were healthy trout taken by rod in the North of Scotland, and one was a healthy trout taken by rod in the Dart in July during the epidemic.

Seven of the 11 experimental trout were sent from the same breeding-station as the seven trout mentioned above which died very rapidly without sufficient apparent reason.

For bacteriological reasons which are given below, this breeding-station cannot be considered above suspicion as regards freedom from the epidemic disease. The experiments by which it was attempted to infect trout with the disease in the laboratory were therefore less satisfactory than was desirable on account of the doubt as to how many of the fish used were infected before the experiment began.

The first four fish which were received (S. 1, S. 2, S. 3 and T. 1) were examined by Dr G. H. K. Macalister, who isolated from them the pigment-forming bacillus which will be described later.

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[1] Dr Masterman informs me that some of these (*e.g.*, two from the River Teign on 12th June) were certainly sea-trout (or peal), though they were thought to be salmon when received in London.

METHOD OF EXAMINATION.

The external naked-eye appearances of the surface of the body and of the internal organs were noted, and bacteriological cultures were made on ordinary neutral or slightly alkaline nutrient agar in Petri dishes. In some cases a special fish-broth agar was used, but it did not appear to have sufficient advantages to encourage its further use. Occasionally neutral-red-lactose-bile-salt agar was used, especially for plating the intestinal contents. The cultures were made from the heart-blood or vena cava-blood, from the liver, spleen, peritoneal fluid, intestinal contents, and mucus exuding from the vent. They were kept at the temperature of the laboratory, which varied from 10° to 22° C. The muscular tissue and any haemorrhages or softened patches discovered in the muscles were also used for inoculating Petri dishes. (In making cultures special care was taken by use of a searing iron to avoid contamination by the skin or other parts of the fish.)

Films for microscopical examination were made from the blood, liver, and spleen, and also from patches of muscular softening. The skin was stripped off, and the interior of the muscles was examined by means of numerous cross-sections. Portions of diseased organs and muscles were hardened and sections examined microscopically.

The examination of the subcutaneous tissues and muscles was not made so completely in the case of the fish examined at the beginning of the investigation as, at that time, its importance was not recognised.

RESULTS OF EXAMINATIONS.

Naked-eye appearances.

Subcutaneous and muscular tissues.—Several of the diseased salmon presented abscess-like cavities under the skin, sometimes of large extent and full of opaque reddish liquid of the consistency of thin human pus. This discharge was often seen to be exuding from one or more small openings in the skin, or the cavity was closed. Some of these cavities extended deeply, laying bare the bones of the fins, and even the ribs, and in some cases the liquid matter had penetrated into the abdomen or thorax between the ribs, a secondary cavity being formed dorsal to the swim-bladder. In addition to these large cavities smaller dark foci were seen which were softened in the centre. These red spots in the muscles were of various sizes, some being as small as a grain of wheat or a pin's head; when very small the spots presented no obvious softening. In no case were the spots and cavities very numerous, five or six being the largest number found in any one fish. The spots of softening and dark red areas closely resembled those described and figured as occurring in the disease known as Furunculosis of the Salmonidae (Emmerich and Weibel, 1894, and Hofer, 1904). The reddish-coloured foci and cavities were met with in any parts of the muscular or subcutaneous tissues, and sometimes they were observed on the head. They were, perhaps, most frequent in the neighbourhood of the lateral line, where the softening and pus-like liquid extended backwards and forwards in the subcutaneous and intermuscular tissues along the lateral line.

No cavities were found in the internal organs, but the liver was often unevenly patchy in colour and sometimes in part softened. The spleen was frequently diffluent and unrecognisable except as a dark red mass with no obvious attachments, looking like a mass of blood clot.

The intestines were frequently congested, but the congestion was not always most intense in the same region. The blood-vessels of the lower part of the gut appeared to be most frequently engorged. Occasionally the pylorus and pyloric caeca were deeply congested. The lower intestine of healthy trout was also sometimes the seat of congestion. As a rule, in the case of diseased fish, blood-stained mucus escaped from the vent when slight pressure was applied to the sides of the abdomen, but this was not quite constant, and occasionally occurred in healthy trout.

Intestinal parasites were not very numerous, but the species of worm which are commonly found in these fish occurred in small numbers. The interior of the swim-bladder areas usually appeared natural, but occasionally one or two nematodes were found.

The gills of the trout appeared healthy; those of the salmon were generally infected with Copepods (*Lernaepoda salmonis*).

Microscopic Pathology.

Films of the puriform matter from the "abscess-like" cavities showed very few cells or nuclei. Bacteria were very numerous and of various forms, chiefly very short oval or oblong Gram-negative bacilli, but longer bacilli were also seen. Films of the heart-blood and from the liver and spleen also showed numerous short bacilli of the same appearance and generally of very uniform size.

Sections of liver, kidney and diseased muscle all presented the same features, viz., patchy necrosis, and disintegration of the tissue cells, no increase in the leucocytes or connective tissue cells, whilst the blood vessels and capillaries were crowded with round or short oblong Gram-negative bacilli of uniform shape and about 1 × 1 or 1 × 1·5 μ in size. Sometimes the red corpuscles in the blood vessels in these diseased areas had almost all disappeared or were represented only by their nuclei.

Bacilli of the same type were also seen in great numbers in the connective tissue spaces in the neighbourhood of the diseased areas, but were absent elsewhere. In the liver similar oblong bacilli were seen in dense clumps of the size of two or three liver cells. Usually but not always, these clumps lay touching the wall of a blood vessel or capillary. In some places bacilli were also fairly numerous in and around the walls of small blood vessels.

Bacteriological Results.

From the diseased fish an easily recognised bacillus possessing well defined characters was obtained constantly and often in almost pure culture from the blood, liver, spleen and peritoneal fluid, and also from the deep red spots in the muscles. The most obvious characteristic of this bacillus was the production of a deep brown diffusible pigment on agar at the temperature of the laboratory. For the present, therefore, this bacillus will be referred to as the "pigment-producing bacillus."

As a rule, other bacteria were very few in cultures from the heart-blood, liver and peritoneal fluid. Those most frequently met with were cocci growing in white, yellow or red colonies. Sometimes also colonies of other bacilli, but in much smaller numbers than those of the pigment-forming bacillus.

On the other hand cultures from the pus of discharging abscesses, the mucus from the vent and the intestinal contents, contained a considerable variety of bacilli. A few colonies of these bacilli were isolated and they proved to be gelatin-liquefying organisms, some of which corresponded in their cultural characters to *B. fluorescens liquefaciens*, and others probably to the *Proteus* group. The latter, however, unlike *B. Proteus vulgaris* fermented mannite with production of gas.

Cultures from the blood and liver of healthy fish yielded as a rule only a few opaque colonies of cocci and moulds, or were sterile.

Two of the healthy control trout which were received dead from the North of Scotland were put in a bowl of tap water in the laboratory two days before they were examined, in order to compare the cultures which resulted with those obtained from other fish which did not arrive in a fresh condition. The organs yielded many more colonies than those of the trout from the same source which were examined at once. All the colonies were apparently of the *proteus* or *fluorescens* type of bacteria, and bacilli producing brown pigment were not found.

THE PIGMENT-FORMING BACILLUS.

Facts as to its occurrence.

This pigment-forming bacillus was found in all the diseased fish except the bream, *i.e.*, in 13 salmon, seven trout and one grayling. It was not found in any of the eight healthy trout from the north of Scotland, nor in the one healthy Dart trout, nor in six out of the seven dead control trout sent from the breeding-station. This bacillus was however found in one (Trout 9) of these seven trout from a fish-culture tank, but in this case only in very small numbers, three colonies appearing on the agar plate inoculated with heart-blood.

Of the 11 fish which were received alive at the laboratory, four which were bought in London gave negative results *post-mortem* as regards this bacillus. The remaining seven fish were none of them quite satisfactory for experimental purposes as some of them were probably infected before they were received. Attempts were made to infect four of them with material known to contain the bacillus. One of the seven fish died soon after arrival without having been infected in the laboratory, and yielded cultures of the bacillus. The remaining two also gave cultures of the bacillus after having been fed with part of a dead trout (Trout 17) from which, however, the bacillus was not isolated, although an attempt had been made to infect it with a pure culture.

However, of these seven fish which were received alive and which all yielded *post-mortem* cultures of the bacillus, only one (Trout 19) was certainly not infected in the laboratory. These seven fish came from the same breeding-station from which was sent Trout 9, which also was found to contain the bacillus, but in small numbers.

This pigment-forming bacillus occurred with such regularity, and, as a rule, in such preponderant numbers in the cultures from the blood, &c., of the *diseased* fish that its characters will now be described at greater length.

It was no doubt identical with *B. salmonicida* Emmerich and Weibel and *B. truttae* Marsh, but at the time of the investigation the modified description of the former of these bacilli by Plehn (1911) had not been published and *B. truttae* Marsh was only reported from a culture tank in America and no description of the disease in which it occurred was available.

Characters of the Pigment-forming Bacillus.

The pigment-forming bacillus which was found with such regularity in the fish which died during the epidemic under consideration shewed the following characters.

Morphology.

In young cultures it was a small Gram-negative bacterium either round, oval or oblong, the long axis being equal to or at most twice as long as the short axis. In older cultures, however, and especially in broth cultures, a few long bacilli occurred which were four or five times as long as they were broad. The diameter of the coccal forms was about 1 μ or rather less.

No true motility was observed though the bacillus was examined at many different stages of growth.

When stained with Loeffler's blue or Carbol-thionin blue or with Leishman's stain, bacillary forms shewed a tendency to bipolar staining.

Cultural Characters.

Growth did not occur or was extremely scanty at 36° C. and agar cultures died at this temperature in two or three days. The optimum temperature for growth appeared to be about 18° to 20° C., but growth did not altogether cease at 5° C.

Ordinary nutrient agar which was slightly alkaline to litmus, gelatin, peptone beef-broth and peptone water all proved good culture media. An agar medium made with fish-broth was also used and gave a more vigorous growth than ordinary agar.

Fairly good growth took place on 0·5 % bile salt agar.

The first cultures direct from the fish were made on ordinary agar, and were grown at the temperature of the laboratory. In some cases the colonies were hardly visible in the first 48 hours. As a rule very small transparent colonies appeared after 24 hours, and in another day or two they had become opaque and about 1 mm. in diameter. The surface of the colonies was moderately shiny and the shape slightly raised and flattened. After two to four days, if the culture was fairly pure and the colonies numerous, the characteristic pigment began to appear. It was of a deep brownish colour and diffused into the agar which remained transparent. In the course of the next week or two the colour deepened till the agar resembled strong black coffee in colour. The time of appearance of the pigment varied considerably and no doubt was very much dependent on the reaction of the medium and perhaps on other peculiarities in its composition. On one occasion when only three colonies of the bacillus were present with a few colonies of other bacteria, no pigment was seen for seven days. When colonies of other bacteria were numerous (*e.g.*, bacilli of the same group as *B. proteus vulgaris* or *B. fluorescens liquefaciens*) the brown pigment often did not appear at all though the pigment producing bacteria were present in considerable numbers. This occurred usually in the case of plates spread thickly with material from the discharging abscesses. The first plate spread would shew no brown pigment, but the second plate spread with the same rod and which contained fewer colonies shewed the brown pigment round some of the colonies.

On the other hand sometimes plates which were crowded with almost pure cultures of the pigment-forming bacillus remained without pigment for five days or more, and then the pigment appeared first round a colony of a coccus or a mould and gradually spread thence and coloured the whole plate. This appearance was probably due to the production of alkali by the contaminating colony.

Subcultures on agar generally shewed good confluent growth in 24 to 48 hours, and pigment in three to four days.

In seven to ten days the colonies themselves as a rule became a uniform pale brownish or buff colour, but occasionally they shewed a deeper brown area in the centre. The time of appearance of the diffusing pigment depended very much on the initial reaction of the agar. The pigment was produced most readily on agar with a reaction of -4 to $+8$ acid to phenolphthalein (Eyre's scale) and it did not appear when the reaction was -6 or $+16$ in one experiment made to test this point. Growth however still took place in broth of -6 and $+16$, but not of -8 nor at $+20$ acidity.

In peptone beef broth the pigment appeared very slowly, and the degree of acidity at which pigment was produced varied within narrower limits.

In peptone water (Witte 2 per cent.) the pigment was produced more readily than in broth, but it did not appear if the reaction was + 20, though pigment was noticed after a week when the initial reaction was + 16.

The pigment was soluble in water, but not in absolute alcohol, chloroform or ether. If absolute alcohol was added to an agar slope some of the pigment diffused out into the alcohol, but the pigment obtained dry from a watery solution was quite insoluble in absolute alcohol.

Indole was not produced in broth cultures.

On gelatin the bacillus grew fairly well, liquefaction usually commencing within the first 24 or 48 hours. Pigment was not usually formed in gelatin cultures, though with an initial acidity of + 15 (Eyre's scale) a slight brown colour was obtained in a week. Gelatin stab cultures shewed a funnel-shaped tract of liquefaction along a course of the needle, and some bubbles of gas were also formed in most cases. On potato the growth was very slight. Cultures made on solidified horse serum grew readily, while the medium became a deep brown colour and was slowly liquefied in 7 to 14 days.

Carbohydrate reactions.

All the strains of the pigment-forming bacillus which were isolated gave identical results when grown on certain carbohydrates in one per cent. peptone water coloured with litmus. The carbohydrates used were glucose, cane-sugar, lactose, mannite and dulcite, and they were added in a proportion of one per cent. to the peptone water. The cane-sugar, dulcite and lactose tubes became alkaline, but in about a week some pigment was produced in these tubes, especially in that containing dulcite, where the blue colour of the medium changed to a reddish tint, though the liquid was still distinctly alkaline to litmus paper. In the glucose and mannite tubes acid and gas were produced in 24 to 48 hours. The amount of gas produced in the case of the glucose was much less than that formed in the mannite tube, as judged by the height of the column of gas in the "Durham's tube." In some rare instances the amount of gas formed in the glucose tube was so slight as to be barely recognisable, none appearing in the small gas tube even after a week's growth. It often happened that no gas was visible in the glucose tube after 24 hours' growth though the acid production was quite distinct.

A variant of the bacillus was found in a colony picked off an agar plate containing chloracetic acid 0·5 $^0/_0$. This variant produced acid without gas from glucose and mannite, in this respect somewhat resembling the variant forms of *B. coli* etc. produced on this medium by Penfold (1911). It is also of interest because it formed very little pigment, like *B. salmonicida* of Emmerich and Weibel and *B. salmonicida-A.* of Plehn. The growth of this variant strain was less vigorous than that of the parent strain.

Viability.

As has been stated already growth continues to go on slowly at 5° C. and sometimes for a short time to a very slight extent at 35° or 36° C. At 37° C. the culture soon dies. Cultures remain alive at 1° C. for at least four months.

From sterile tap water the bacilli could be recovered alive, in slightly diminished numbers, after five days. The experiment was made by emulsifying an agar culture in 100 c.c. of tap water and leaving the flask at room temperature. Two or three drops were removed and spread over the surface of a solid agar plate at varying intervals. An agar culture was also emulsified in the same way in 100 c.c. of sterilised *sea water*. In this case the bacilli rapidly died. After two hours the number of colonies which grew from a drop taken from the flask containing sea water was reduced from several hundred at the beginning of the experiment to 80, and after 19 hours no growth occurred. From a mixture of sea water and tap water in equal parts none of the bacilli could be recovered after 19 hours, but from a mixture containing sea water 25 per cent. and tap water 75 per cent. a fair growth was obtained after 45 hours, but not after 67 hours. Salt water (3 per cent. sodium chloride in distilled water) also had a destructive effect on the bacilli. The growth from one agar slope was emulsified in 500 c.c., of this 3 per cent. salt water. One drop taken after two days gave only one colony on an agar plate. On the other hand a flask containing an agar culture in 500 c.c. of tap water, when examined after two days, yielded unaccountable colonies from a drop plated in the same way.

Agglutination.

Emulsions of this bacillus made with normal salt solution (0·85 per cent.) very soon shewed complete clumping of the bacilli, and the same thing occurred if 0·2 per cent. salt solution was used. An emulsion in distilled water remained unclumped for 24 hours, and occasionally an emulsion from a culture on agar of acidity + 20 (Eyre's scale) remained uniform in 0·85 per cent. salt solution.

Some agglutination experiments were made with emulsions in distilled water, but no distinct agglutination was obtained with the serum of diseased fish in higher dilution than 1 in 10.

Pathogenicity.

The experiments were not conclusive as the supply of healthy living trout or salmon available for experiment was not sufficient for the purpose.

The following experiments were, however, performed :—

Altogether 11 trout were kept alive in the laboratory and examined after death. Of these, four healthy trout were bought in London, and after they had lived three or four days in running water in the laboratory they were examined. These trout are numbered 15 to 18 inclusive.

T. 15.	Control ...	21st June. Killed.
	Post-mortem ...	Pigment-forming bacillus not found.
T. 16.	Control ...	21st June. Killed.
	Post-mortem ...	Pigment-forming bacillus not found.
T. 17.	19th June ...	Attempted infection by feeding and the addition of half an agar culture to the bowl.
	21st June ...	Died.
	Post-mortem ...	Pigment-forming bacillus not found.
T. 18.	19th June ...	Attempted infection by the same means.
	21st June ...	Died.
	Post-mortem ...	Pigment-forming bacillus not found.

Seven trout were received alive from a breeding station. These are numbered 19 to 23 inclusive, 32 and 33.

T. 19.	Control ...	No attempt at infection.
	23rd June . .	Died.
24th June.	*Post-mortem* ...	Almost pure culture of the pigment-forming bacillus obtained from the blood and liver.
T. 20.	21st June ...	Side was scratched and the scratch inoculated with a pure culture.
	23rd June ...	Died.
24th June.	*Post-mortem* ...	Much growth of the pigment-forming bacillus obtained from blood, liver and spleen.
T. 21.	21st June ...	Fed with liver of T. 17.
	24th June ..	Died.
24th June.	*Post-mortem* ...	Almost pure cultures of the pigment-forming bacillus.

T. 22.	21st June ...	Fed with liver of T. 17.
	26th June ...	Died.
26th June.	*Post-mortem* ...	Almost pure culture of the pigment-forming bacillus from blood.
T. 23.	21st June ...	Scratch on side and pure culture put in water.
	25th June ...	Died.
	Post-mortem ...	Pigment-forming bacillus cultivated from blood.
T. 32.	5th July ...	Attempted infection by feeding with pure culture.
	14th July ...	Attempted infection by adding pure culture to water.
	15th July ...	Attempted infection by adding pure culture to water. Fish remained well.
	27th Sept. ...	Scratch on side and pure culture rubbed in.
	30th Sept. ...	Died.
	Post-mortem ...	Local redness of skin and sloughy softening of tissues around wound. Many colonies of pigment-producing bacillus from heart-blood.
T. 33.	19th June ..	Attempted infection by adding pure culture to water.
	5th July ...	Attempted infection by adding pure culture to water.
	14th July ...	Attempted infection by adding pure culture to water.
	15th July ...	Fish died from an accident.
	Post-mortem ...	Pigment-forming bacillus found in blood, &c.

Five gold-fish were used experimentally. Two (Gf. 5 & 6) were inoculated with 0·25 c.c. of broth culture intraperitoneally. They both died in about 24 hours and the bacillus was recovered from the blood.

Gf. 1.	13th June ...	Received half an agar slope of a pure culture in water.
	5th July ...	Scratch on side and pure culture rubbed into wound.
	10th July ...	Injured region of skin has lost its scales and has a surrounding area of redness. Recovery.
	10th October...	0·2 c.c. of an emulsion of a pure culture from Trout 7 (one loop emulsified in 1 c.c. of water) injected into muscles near dorsum.
	12th October...	Swelling, desquamation and redness around.
	14th October...	Killed.
	Post-mortem ...	Pigment-forming bacillus found in spleen and muscles.
Gf. 2.	13th June ...	Attempt to infect by placing a piece of intestine of Salmon 4 into water.
	30th June ...	Attempt to infect by putting pure culture in water. Remained well.
	10th October...	Intramuscular injection of 0·1 c.c. of the same emulsion as that given to Gf. 1.
	12th October...	Swelling.
	14th October...	Swelling increasing, blood red ring on skin around swelling.
	18th October...	"Abscess" discharging. Died (8 days after injection).
	Post-mortem ...	Large cavity extending deeply amongst muscles. Pigment-forming bacillus found in "abscess" and spleen.
Gf. 3.	Attempt to infect by keeping in same bowl as Gf. 2 which had a discharging "abscess." Remained healthy for over three weeks.	
	10th November	Killed. Pigment-forming bacillus not found.

From these imperfect inoculation experiments it appears that it is possible to infect trout by scratching the side and rubbing in pure culture, and gold-fish by subcutaneous or intramuscular injection of small quantities of culture. In both cases cavities full of puriform matter are produced locally as in the natural disease.

Evidence of Pathogenicity.

In the absence of further attempts to produce the disease with pure cultures it is impossible to affirm, as far as this investigation is concerned, that the pigment-producing bacillus is the cause of the disease, but there is presumptive evidence that this is the case.

(1) The pigment-producing bacillus was present in 21 out of 22 diseased fish examined, *i.e.*, in 13 salmon, seven trout, and one grayling, but was not found in one bream sent up for examination.

(2) It was present in large numbers, and often almost in pure culture, in the blood from the heart or vena cava, and in the liver and spleen of these 21 diseased fish.

(3) It was absent from 13 healthy control trout and one control salmon. The salmon as well as one of the trout came from an infected river.

(4) From only one source, viz., a trout-breeding station, were fish which were sent as healthy controls found to be infected. Out of seven control fish which were sent dead from this source, only one yielded a very few colonies.

The seven control fish which were sent alive all eventually gave cultures of the pigment-forming bacillus, but only one of these certainly, and two others probably, had not been infected in the laboratory.

Therefore of ten fish from this doubtful source, which, however, were regarded as healthy controls, four yielded cultures of the bacillus.

(5) To sum up the results as regards healthy controls, out of 23 fish which were regarded as healthy controls, only four yielded cultures of the bacillus. These cultures all came from fish obtained from a single source, namely a fish-breeding station, and two of these fish were possibly infected in the laboratory.

(6) Laboratory experiments show that this pigment-forming bacillus is capable, when injected intramuscularly or intraperitoneally, of producing an "abscess" in gold-fish followed by septicaemia and death, and when inoculated into a trout through a scratch can produce deep ulceration, septicaemia, and death.

It remains to be shown whether this bacillus will infect trout or salmon with any degree of certainty when the bacillus is introduced into the water in which the fish are living, and whether a wound is necessary for the entrance of the bacillus. The experiments of Emmerich and Weibel, and of Plehn appear to show that the contamination of the water with a culture of the bacillus is sufficient to infect uninjured fish.

Remarks.

There seems to be no doubt that the epizootic in this country was due to the disease known on the continent as Furunculosis of the Salmonidae. This disease as has been pointed out by Emmerich and Weibel (1896), Hofer (1904), and Plehn (1911) is not always characterised by the muscular haemorrhages and foci of softening in the muscle to which its name is due. Apparently the fish frequently die at an early stage of the disease before localisation of the bacteria in the muscles has occurred and whilst no pathological change except septicaemia is discernible.

The strain of bacillus isolated from English fish almost exactly resembles that described by Marsh (1903) and called by him *B. truttae*. The only points of difference in his description are (1) his statement that acid is produced from glucose, but that this sugar is not fermented. This statement must be taken to mean that acid is produced but no gas. The English bacillus produced only a small amount of gas and sometimes this was so slight as to be unnoticed for two or three days. The variant mentioned above which occurred on a chloracetic acid agar plate and only produced a very small amount of pigment, formed no gas from either glucose or mannite. The production of neither acid nor gas from cane sugar is a character in which the English bacillus and Marsh's agree. (2) The brown pigment formed is said by Marsh to be soluble in alcohol. This was not found to be the case as regards the pigment formed by the English bacillus when the pigment was procured in a dry form. It was found however that if absolute alcohol was poured on to a moist pigmented agar culture, the watery solution diffused out into the alcohol and the pigment remained dissolved in the diluted alcohol.

Bacillus salmonicida of Emmerich and Weibel must be regarded as identical with *B. truttae* of Marsh and *B. salmonicida-B.* of Plehn and also with the English bacillus described in this paper. The German observers have not recorded the results of any carbohydrate tests and the originally described character of liquefaction of gelatin with the formation of gas, which was considered as distinctive in the original description, is not sufficient to separate the bacillus from some of the Gram-negative non-motile bacilli which are found in the intestines of fishes. The very characteristic production of brown pigment is dependent on a correct reaction of the culture media, but was almost always obtained in my cultures with ordinary nutrient agar containing Witte's peptone and beef broth. Apparently, however, certain other less readily controlled conditions are necessary for a satisfactory and rapid pigment production. On one occasion ten or twelve of my stock cultures failed to form pigment on agar.

The distinctive characters of *B. salmonicida*, *B. truttae* and *B. salmonis pestis* are shown in Table IV. as well as the characters of some of the other bacilli found most frequently on the cultures made from the diseased fish.

CONCLUSIONS.

1. The epizootic disease which occurred amongst salmon and trout in the south-west of England during the summer of 1911 presented the same symptoms as the disease which occurs in Germany, especially Bavaria, and in Austria and France, and is called Furunculosis of the Salmonidae.

2. The most characteristic feature of the disease, *i.e.*, abscess-like cavities, softened haemorrhagic foci, and small haemorrhages in the muscles were of frequent occurrence, especially in the larger salmon, but also were frequently absent.

3. Congestion of the intestine, especially the lower intestine, and discharge of blood and mucus from the vent *post-mortem* were of constant occurrence.

4. No bacteriological evidence was obtained that fish other than members of the family of Salmonidae were attacked.

5. A pigment-forming bacillus occurred constantly in large numbers in the blood and internal organs and in the muscular haemorrhages of fish dying of the disease, but it was absent from the very large majority of healthy control fish.

6. This bacillus is probably identical with *B. salmonicida* described by Emmerich and Weibel, as the cause of Furunculosis of the Salmonidae, and with *B. truttae* described as causing an epidemic amongst trout in America.

7. It is almost certain that this bacillus is the cause of the epizootic disease.

8. This bacillus was present in some apparently healthy fish which had been bred in captivity. These infected fish may have been examined during the incubation stage of the disease, or before the symptoms became noticeable, or they may have been healthy carriers of the bacillus.

9. The bacillus associated with this epizootic was destroyed in a few hours by sea-water, and even by a mixture of equal parts of sea-water and tap-water, although it lived for at least five days in tap-water. It is therefore improbable that salmon usually become infected in the sea.

10. The infection probably spreads from fish to fish in fresh water. The spread is no doubt facilitated by conditions of low water.

11. Cultures of the bacillus soon died out at a temperature of 36° C. and therefore it is extremely improbable that this bacillus ever attacks warm-blooded animals.

12. The especially high mortality from the disease during hot weather and the experiments of Plehn suggest that a raised temperature of water favours the development of the disease, when *B. salmonicida* is already present.

The work on the epizootic disease of the Salmonidae (Furunculosis) which has already been accomplished leaves much still to be done before the natural history of this disease is understood sufficiently to control future outbreaks in rivers.

The following points especially require further investigation:—

1. Whether salmon coming up from the sea are infected before they enter the rivers.

2. Whether the spread of the disease from river to river is only due to the introduction of fish from other rivers and breeding stations.

3. Whether other fish besides the Salmonidae (salmon, salmon trout, trout, grayling, and rainbow trout) are naturally infected.

4. Whether salmon and trout frequently harbour the bacillus for long periods though apparently in good health, and whether any such infected fish survive the cold weather in the rivers, and so carry on the infection from year to year.

5. Whether infection is more easily obtained by feeding or by pollution of the water with the bacillus, and, if the latter, whether wounds are necessary as a predisposing cause for the infection.

6. Whether infected fish not suffering from discharging ulcers can readily communicate the disease to others.

7. The effect of the temperature of the water on the production and prevalence of the disease.

Though widespread, the distribution of this disease does not appear to be universal at present. Fresh rivers are probably infected by the introduction of fish from infected sources. By careful attention to this means by which the disease is spread, the infection of fresh waters might perhaps be avoided.

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TABLE I.

No.	Fish	Date	Source	Diseased or Control	Post-mortem	Organs from which pigment-forming bacillus cultivated	Pure culture
S. 1	Salmon ...	20. v. 11	Exe ...	Diseased	—	Blood ...	+
S. 2	,, ...	1. vi. 11	,, ..	,,	Haemorrhage in peritoneum	Blood, liver, muscle	+
S. 3	,, ...	3. vi. 11	Wye ...	,,	Haemorrhage in peritoneum	Peritoneal fluid	+
S. 4	,, ...	13. vi. 11	,, ...	,,	(Viscera removed) ...	Blood ...	-
S. 5	,, ...	13. vi. 11	Teign...	,,	Bloody fluid from vent	Blood, liver, gut, spleen	+
S. 6	,, ...	13. vi. 11	,, ...	,,	Bloody fluid from vent	Peritoneal fluid	+
S. 7	,, ...	13. vi. 11	,, ...	,,	Small ulcers of skin. Haemorrhage in peritoneum	Blood, peritoneal fluid	+
S. 8	,, ...	14. vi. 11	Exe ...	,,	(Dried up)	Blood ...	+
S. 9	,, ♂ 2 ft. 6 in.	28. vi. 11	Wye ...	Control	Bloody fluid from vent	—	
S. 10	Salmon ♀ 3 ft. 3 in.	29. vi. 11	,, ...	Diseased	Large abscess ...	Blood abscess, peritoneal fluid	+
S. 11	Salmon ♂ 3 ft. 6 in.	7. vii. 11	Erwood	,,	Abscesses	Blood, muscle, peritoneal fluid	+
S. 12	Salmon ♂ 2 ft. 9 in.	18. vii. 11	Dart ...	,,	Abscesses. Haemorrhage in muscles	Blood, muscle	+
S. 13	Salmon ...	1. viii. 11	? ...	,,	Abscesses	Blood ...	+
S. 14	,, 2 ft. 7 in.	3. viii. 11	Dart ...	,,	Abscesses	Liver	+
F. 1	Grayling ♀	18. vi. 11	Wye ...	,,	Blood from vent. Fluid in peritoneum	Peritoneal fluid	+
F. 2	Bream ...	18. vi. 11	,, ...	,,	—	—	-

TABLE II.

Ref. No.	Fish	Date	Source	Diseased or Control	Post-mortem	Organs from which pigment-forming bacillus cultivated	Pure culture
T. 1	Trout ...	1. vi. 11	? ...	Diseased	Macerated	Liver	+
T. 2	,, ...	10. vi. 11	Exe ...	,,	Fresh in ice ...	Blood ...	+
T. 3	,, ...	,,	,, ...	,,	Congestion round vent; in ice	Blood ...	-
T. 4	,, ...	,,	,, ...	,,	In ice	Blood, liver ...	+
T. 5	,, ...	,,	,, ...	,,	In ice	Blood ...	+
T. 6	,, ...	,,	,, ...	,,	In ice	Blood	-
T. 7	,, ...	,,	,, ...	,,	Bloody fluid from vent; in ice	Blood, liver, gut	-
T. 8	,, ...	13. vi. 11	Fish Culture Tanks	Control	Nil		-
T. 9	,, ...	,,	,,	,,	Bloody fluid from vent	—	
T. 10	,, ...	,,	,,	,,	Nil	—	
T. 11	,, ...	,,	,,	,,	Nil	—	-
T. 12	,, ...	,,	,,	,,	Bloody mucus from vent	Blood (three colonies)	+
T. 13	,, ...	,,	,,	,,	Bloody mucus from vent	—	
T. 14	,, ...	,,	,,	,,	Nil	—	
T. 24	,, ...	1. vii. 11	Scotland	,,	Bloody fluid from vent	—	
T. 25	,, ...	,,	,,	,,	Nil	—	
T. 26	,, ...	,,	,,	,,	Nil	—	
T. 27	,, ...	,,	,,	,,	Nil	—	-
T. 28	,, ...	,,	,,	,,	Nil	—	
T. 29	,, ...	,,	,,	,,	Nil	—	
T. 30	,, ...	,,	,,	,,	Macerated; 2 days in water in laboratory	—	
T. 31	,, ...	,,	,,	,,	—	—	-
T. 34	,, ...	26. vii. 11	Dart ...	,,	Fresh	—	-

TABLE III. (*Experimental Trout.*)

Ref No.	Fish	Source	Experimental or Control	Method of infection	Date of experiment	Date of death	Post-mortem	Organs from which pigment-forming bacillus cultivated	Pure culture
T. 15	Trout	Shop	Control	—	—	21.vi.11	Nil	—	-
T. 16	..	..	,,	—	—	21.vi.11	Intestine congested	—	—
T. 17	..	..	Exp.	Culture in water	19.vi.11	21.vi.11	Intestine congested	—	—
T. 18	..	..	,,	Culture in water	19.vi.11	21.vi.11	Intestine congested	—	—
T. 19	..	Fish culture tank	Control	—	—	24.vi.11	Petechiae on swim bladder	Blood, liver	+
T. 20	,,	,,	Exp.	Scratch on side inoculated pure culture	21.vi.11	24.vi.11	Intestine congested, large spleen	Blood, liver spleen	+
T. 21	,,	,,	,,	Fed with liver of T. 17	21.vi.11	24.vi.11	Inflamed spot on side	Liver, spleen blood, local lesion	+
T. 22	,,	,,	,,	Fed with liver of T. 17	21.vi.11	25.vi.11	Nil	Blood, liver, spleen	+
T. 23	,,	,,	,,	Scratch on side and culture in water	21.vi.11	25.vi.11	Inflamed wound, patches of congestion on liver	Blood, spleen, wound	+
T. 32	,,	,,	,,	Scratch and pure culture rubbed in	27.ix.11	30.ix.11	Large discharging wound	Blood, liver, wound	+
T. 33	..	..	..	Water infected	19.vi.11	15.vii.11	Nil	Blood ...	+

TABLE IV.

Bacillus	Morphology	Gram staining	Motility	Agar	Potato	Serum	Glucose	Cane-sugar	Lactose	Mannite	Dulcite	Indole	Milk	Growth at 37° C	Remains alive in sea water	Anaerobic (facultative)	Spores	Gelatine
B. salmonicida ... ... (England, 1911)	Short	–	–	Brown diffusing pigment	Very slight	Lique-faction deep brown	Acid and gas	–	–	Acid and gas	–	–	Acid digestion	Very slight	A few hours	+	–	+
B. truttae ... .. (Marsh)	Short	–	–	Brown diffusing pigment	Very slight	Lique-faction deep brown	Acid	–	–	..	...		Acid digestion	None	..	Slight	–	+
B. salmonicida ... ... (Emmerich and Weibel)	Short	–		Slight late brown pigment	None	...	...	–	...	..	...	...	..	None	...	+		+
B. salmonis pestis ... (J. Hume Patterson)	Short	–	+	None	Profuse yellowish brown growth	No lique-faction	...	...	...	...	...	...	Acid digestion	Very slight	One month	–		+
Bacillus of Proteus group (Fish, 1911)	Irregu-lar and long	–	+	None	Scanty	...	Acid and gas	Acid and gas		Acid and gas		+	Digested deodorised	+	...	+	–	+
B. fluorescens liquefaciens (Fish, 1911)	Long	–	+	Green diffusing pigment	Yellow	..	Acid	–			–	+ slight	Digested deodorised	+	...	–	–	+

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NATURAL VARIATION OF *B. ACIDI LACTICI* WITH RESPECT TO THE PRODUCTION OF GAS FROM CARBOHYDRATES.

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Introduction.

Variations in bacteria may be said to fall into two classes (1) those which are temporary, and dependent on the environment, the bacteria tending to return to the original type when subculture is made on ordinary media; and (2) those which are fixed, and persist after repeated subcultures on ordinary indifferent media.

Although, for most purposes, this classification is useful, it is not always easy to decide to which class a given variety belongs. The altered culture may rapidly revert to the original type, because it consists of a mixture of the new variant and the original form, and ordinary media may exercise a selection which rapidly reduces the new variant to an imperceptible minority, or may lead to its complete extermination; while on the other hand growth on the medium on which the variant first appeared, favours the new form.

There may be a mixture of varieties of this kind in a culture which springs from a single variant bacillus, for though the majority of the bacilli produced belongs to the new variety, a small proportion of the original type often occurs by reversion in the course of successive generations.

That the proportion of variants in a culture depends on the medium on which the culture has been made and on the age of the culture was shown by Penfold (1911) in the case of cultures of the *Bacillus typhosus* in dulcite-peptone water in which the proportion of bacilli capable of fermenting dulcite increased progressively.

Penfold also found that the variety of *B. coli* which had lost the power of producing gas from sugars after growth on chloracetic agar, did not remain constant till selection on the latter medium had been repeated. This tendency to revert in the earlier phases of the variant culture, indicated the presence of a mixture of the individuals which produced gas, and of those which did not, and perhaps also of intermediate variants.

The intermediate strains of the bacillus whose occurrence is about to be recorded in this paper, appear to have consisted of similar mixtures, though derived from colonies which in all probability arose from single bacilli.

Only that variety which remains constant on ordinary culture media can be considered as a true fixed variety, but even in this case the constituents of the medium may have a special selective action in some direction, and unless no atavist variant bacilli make their appearance in the course of growth the strain may revert to the original type.

The tendency of a variant to revert in culture, therefore, depends on the occurrence of individual bacilli of the original type and also on the favouring of these forms by the culture medium, but, frequently, atavistic bacilli may occur without an obvious change in the culture because the medium does not afford them any preferential treatment. On the other hand, if the particular medium is strongly selective for any particular type, the culture will in time take on the character of this type if individuals of this variety occur, even in small numbers.

Variations which remain constant in artificial culture have been described affecting many of the characters and biochemical properties of bacteria, but I propose here to mention only those relating to the fermentation of the carbohydrates.

Variations in fermentation.

Among the characters which are used for distinguishing allied forms of bacteria of the coli-typhoid group the production of acid, or acid and gas from sugars and alcohols, has proved to be of great service since these characters are as a rule uniform for known pathogenic bacilli both when the organism is first isolated and even after long growth and repeated subcultures on ordinary laboratory media.

Variations in fermentation reactions are not only of practical importance for diagnostic purposes, but are also of great theoretical interest, since they affect some of the characters, which have usually been reckoned among the more permanent features of bacteria, and concern the physiological processes intimately connected with the life and growth of the bacteria.

Varieties showing changes in the fermentation characters during artificial culture have been described and fully tested of recent years.

Hiss (1904) found that *B. dysenteriae* Type *Y* could acquire the power to ferment maltose by growing on a medium containing that sugar.

Twort (1907) produced similar changes in *B. typhosus* as regards dulcite and lactose.

Lentz (1909) and others have made similar observations.

Arkwright (1909) recorded natural and cultural changes in the fermentation properties of the meningococcus, which occurred independently of growth on sugars.

Penfold (1911, 1912) studied variations of this type in detail and has summarised the work on bacterial variation generally. He also confirmed and extended the work of Massini (1907) on *B. coli mutabile.*

Baerthlein (1912) correlated changes in the sugar reactions with variations in the type of colony on agar plates.

These examples, from amongst a large number of observations, illustrate for the most part the variations which have been observed in the direction of acquiring additional characters.

Other observations, which are no less striking, have been made on the loss or partial suppression of some characters.

Scheierbeck (1900) showed that streptococci occurring in milk vary very much in the amount of acid which they produce in a given time, and he was able by growing cultures in milk containing carbolic acid to obtain strains with widely different acid-forming powers, which remained constant after frequent subculture.

Penfold (1911, 1912) showed that it was possible to suppress to a very large extent the gas-forming property of *B. coli, B. enteritidis* (Gaertner) and *B. acidi lactici* by growing these organisms on agar containing chloracetic acid. The new variety in this case while forming acid from the same sugars and alcohols as the original strain had lost the property of forming gas from sugars, but retained it for the alcohols. He proved the identity of the two varieties by means of serological tests. The variants remained constant through numerous subcultures.

Revis (1911) by growing *B. coli* (Escherich) on 0·1 % malachite green broth succeeded in suppressing the gas-forming function for alcohols and sugars, the variant still producing acid from the same sugars and alcohols as the original strain.

Natural variations.

Reference to the writings of workers on the subject of variation of bacteria, shows that while under special conditions definite and fixed varieties can be observed to occur, still there is little direct evidence to show that similar variation takes place under natural conditions, *e.g.* in the human body or in the outside world.

Colonies of the same organism but of dissimilar appearance occasionally appear on the original plates used for isolation, but when subcultures are made these differences usually disappear.

There is however some evidence (Baerthlein 1912) that fixed varieties which can be distinguished by the appearance of the colonies may be observed on the original plates inoculated with the stools of a patient.

The proof that these different types of colony consist of the same organism, is often imperfect, and in many of these cases proof of recent origin from a single strain is impossible and the same patient may have been infected from two different sources.

The acid-producing variety of *B. coli mutabile* which first appears on lactose agar subcultures has not as yet been conclusively demonstrated in the faeces. It is therefore only known to arise under artificial conditions.

Penfold (1912) however showed that the lactose-fermenting variants which are produced by *B. coli mutabile* closely resemble those lactose fermenters which commonly occur in the faeces.

It is more frequently in later subcultures that variations have been noticed, and since the best evidence of variation has been obtained by dealing with pure cultures from a single colony, most attention has been turned in this direction.

The natural varieties of pathogenic bacteria which may be isolated from different patients or in different epidemics, *e.g.* the morphological varieties of the *B. diphtheriae* described by Dale (1910) and the well-known subgroups of the mannite-fermenting type of the *B. dysenteriae*, belong to a different category, since it cannot be known how long their special characters have been acquired nor whether they first appeared in the patient from whom they were isolated.

In this connection must be mentioned the two following observations:

Bock (1906) found that three strains of *B. suipestifer* from different laboratories formed no gas from glucose, but as regards agglutination behaved like normal strains. He used malachite green agar ($\frac{1}{400}$ and $\frac{1}{500}$) for differentiation but does not state whether these strains had grown on this medium.

Bainbridge (1909) examined a bacillus which had been sent to him as a strain of *B. suipestifer* and found that it never formed gas from carbohydrates, although it formed acid from the same media as other strains, and in all other respects, including agglutination, resembled the standard *B. suipestifer*.

Nothing is known of the circumstances under which these varieties have arisen.

When bacteria have been met with, which present characters suggesting that they are natural variants of well-known types, direct evidence of the origin of the variant from the type form is usually absent or unconvincing. For instance, strains of bacteria belonging to the coli-typhoid family which resemble members of the *B. coli* group in producing acid from lactose, and in not liquefying gelatine, but which produce no gas from the sugars from which acid is formed, have been described by a few writers.

Mair (1906) recorded two strains of a coliform bacillus isolated from the urine of two patients which formed acid but no gas from glucose and lactose.

Wilson (1908) retested Mair's strains. He also examined 50 varieties of coliform organisms from the urine of cases of cystitis, pyelitis etc. Three of these latter strains formed acid but no gas from glucose. Including Mair's two bacilli he investigated five such strains. Of these five, two formed acid from the same sugars and alcohols as *B. acidi lactici*, with the exception of adonite, and also like it formed indole in broth. One of the two latter strains formed acid and gas from mannite and sorbite, but only acid from glucose, lactose and other sugars, and therefore resembled Penfold's chloracetic variants.

I have been able to find a record of only one instance in which two varieties of a bacillus, which differ in little except in the production and non-production of gas, have been isolated from the same patient under circumstances which make it practically certain that the two varieties are in reality of identical origin and have become differentiated in the body of the patient.

The case referred to is that described by Sörensen (1912) who at different times isolated two strains of a coliform bacillus from the urine of a glycosuric patient. The two strains gave identical cultural reactions except that the one (Strain I) formed gas from all the sugars and alcohols from which acid was produced, and the other (Strain III *a*) formed acid only and no gas from these substances. (See Table I.)

TABLE I.

Sörensen's Bacillus	I	III *a*	III *b*	III *c*	IV
Glucose	A. & G.	A.	A.	A. & G.	A. & G.
Galactose			A.	A. & G.	A. & G.
Fructose			A.	A. & G.	A. & G.
Mannose			A.	A. & G.	A. & G.
Starch			A.	A. & G.	A. & G.
Maltose			A.	A. & G.	A. & G.
Lactose	A. & G.	A.	A. & G.	A. & G.	A. & G.
Cane sugar	A. & G.	A.	A.	A. & G.	A. & G.
Inulin			A.	A. & G.	A. & G.
Raffinose			A.	A. & G.	A. & G.
Mannite			A.	A. & G.	A. & G.
Milk	A.	A.	A.	A. & clot	A. & clot

There was evidence moreover that these two varieties exhibited the same differences in the patient's bladder. On the occasions on which the gas-forming variety (I) was isolated, the patient's bladder contained gas, and when the variety (III *a*) which did not form gas in culture was present, the gas in the bladder was not observed.

Sörensen showed the identity of the two strains by fermentation and cultural reactions but not by serum tests. Some differences were observed in the growth in broth. The gas-former grew with a slimy surface film on the medium and the bacilli grew in long threads, whereas the slimy character was absent in the other variety and the bacilli were shorter.

After growth for a month in artificial culture the variety of bacillus which at first formed no gas suddenly reverted to the gas-forming type (III *c*) as a sequel to growth on a 2 % glucose medium.

At one period a culture (III *b*) with intermediate characters occurred in the course of cultivation on artificial media. This intermediate variety was distinguished by the formation of gas from lactose, but not from glucose or other sugars nor from alcohols.

At a later date the patient's bladder was again found to contain gas, and a gas-forming strain (IV) like Strain III *c* in every particular was isolated.

Strains I, III *c* and IV resembled each other in that they formed a slimy pellicle on broth; III *c* and IV differed from the other strains by clotting milk.

NATURAL VARIATION OF A BACILLUS OF THE *B. ACIDI LACTICI* GROUP.

The bacillus which is the subject of the present communication was isolated from the urine of a man of 79 years who was suffering from an enlarged prostate and a variable degree of cystitis.

This bacillus, which belongs to one of the subgroups of *B. coli*, has been isolated from the urine every time that it has been examined, *i.e.* eight times in the 11 months from Feb. 1912 to 16 Jan. 1913. Though the bacillus presents variations in its characters, it is believed to be essentially the same throughout.

The urine has been passed into a sterile vessel and examined immediately. At first both staphylococci and streptococci were present as well as the coliform bacillus, but no streptococci were found at the last seven examinations and only a few staphylococcus colonies appeared on the cultures.

The deposit obtained by centrifuging the urine was examined in stained films and by plating out on agar. The bacillus was always found in large numbers by both methods.

Characters of the bacillus.

The bacillus isolated at the first examination of the urine in February 1912 had the same characters as the *B. acidi lactici* (Hueppe) as described by MacConkey (1909) except as regards motility. The typical *B. acidi lactici* is non-motile, but the bacillus now described is slightly motile in the sense that a few motile individuals can be found in six hours' and 24 hours' broth cultures. It therefore more accurately corresponds to No. 1 on MacConkey's list. It may perhaps for purposes of classification be called a motile variety of *B. acidi lactici.*

In morphology the bacillus varies in length but in young agar cultures it is frequently very short and almost coccal.

The tests used on each occasion on which it has been isolated have been, growth on gelatin, litmus milk, peptone-water containing 1 % of glucose, cane-sugar, lactose, dulcite, mannite, adonite or inulin, and Ehrlich's test for indole in broth cultures.

On the original agar plates used for isolation, the colonies have usually been very small and translucent for 24 to 48 hours, but later and in subcultures large semi-opaque flat colonies have developed.

On the last seven occasions during the four months Sept. 1912 to Jan. 16, 1913, on which the bacillus has been isolated, several colonies have been examined from each specimen by the above mentioned tests. A few colonies have been grown on a more extended series of carbohydrates, glucosides and alcohols, as will be detailed later. (See Table III.)

The result of these examinations has been that two distinct varieties of the bacillus have been isolated, and in addition cultures showing intermediate characters have frequently been obtained by picking off single colonies from the original agar plates.

TABLE II.

Varieties of bacillus.

	I	II	III	IV
Glucose	A. G.	A.	A.	A. G. S.
Lactose	A. G.	A.	A. G.	A. G.
Cane sugar	–	–	–	–
Inulin	–	–	–	–
Mannite	A. G.	A.	A. G.	A. G.
Dulcite	–	–	–	–
Adonite	A. G.	A.	A. G.	A. G.
Milk	A. C.	A. C.	A. C.	A. C.
Indole	+	+	+	+
Motility	+ S.	+ S.	+ S.	+ S.
Gelatin	–	–	–	–

A. = acid. A. G. = acid and gas. A. C. = acid and clot. + = indole positive. + S. = slight motility. – = no change in reaction of carbohydrates, or no liquefaction of gelatine.

I and II represent the two extreme varieties, III and IV the intermediate varieties.

Colonies on the original plates have, as a rule, been uniform in appearance and, when some difference in size or opacity has been noticed, these differences were not correlated with a different behaviour on test media.

No coliform bacillus giving other reactions has ever been found and the only other bacteria discovered have been staphylococci and previously to September 1912 also streptococci.

As is shown in Table II the only difference between the varieties has been in the production of gas, except perhaps some difference in length of the bacilli and occasionally in the size of the colonies in subcultures.

Cultures of variety I sometimes show a longer form of bacillus than is found in similar cultures of variety II.

The two extreme varieties (I and II) have almost always bred quite true in artificial culture both when frequently subcultured and when left without subculture for upwards of three months on agar.

Evidence of an irregular change in an old agar culture has been noticed once and has latterly been obtained by growth in special media.

The intermediate varieties differed from the extremes only in details of the gas-producing function. They differed somewhat among themselves as regards the same character, some forms producing no gas from glucose but a full amount from lactose, while others produced a very little gas from glucose which was not increased after a week's incubation.

Some colonies produced only a very little gas from mannite.

TABLE III.

	I	Ia	II	A	B	C
Glucose	A. G.	A. G.	A.	A.	A.	A. G.
Laevulose	A. G.	A. G.	A.	A.	A.	A. G.
Galactose	A. G.	A. G.	A.	A.	A.	A.
Inulin	–	–	–	–	–	–
Dextrin	A. G. S.	A. G.?	A. G.?	A. G.?	A. G.?	A. G.?
Maltose	A. G.	A. G.	A.	A.	A. G. S.	A. G.
Lactose	A. G.	A. G.	A.	A.	A. G. S.	A. G.
Cane sugar	–	–	–	–	–	–
Raffinose	–	–	–	–	–	–
Arabinose	A. G.	A. G.	A.	A.	A. G. A.	A. G.
Salicin	A. G.	A. G.	A.	A.	A. G.	A. G.
Amygdalin	–	–	–	–	–	–
Isodulcite	A. G. S.	A. G. S.	A.	A. G.	A.	A.
Mannite	A. G.	A. G.	A.	A.	A.	A. G.
Dulcite		–	–	–	–	–
Sorbite	A. G.	A. G.	A.	A.	A. G. S.	A. G.
Adonite	A. G.	A. G.	A.	A.	A. G. S.	A. G.
Erythrite	A.?	–	–	–	–	–
Inosit		–	–	–	–	–
Glycerine	A. G.	A. G.	A.	A.	A. G.	A. G.
Milk	A. C.	A. C.	A. C.	A. C.	A. C.	
Indole	+	+	+	+	+	+
Motility	+ S.	+ S.	+ S.	+ S.	+ S.	+ S.
Gelatin		–	–	–	–	–

A = acid. A. G. = acid and gas. A. G. S. = acid and slight gas. A. C. = acid and clot. Indole + = indole formed. Motility + S. = slight motility. Gelatine – = no liquefaction.

Moreover these intermediate forms showed a great tendency to change into variety I which produced a full amount of gas, or occasionally into variety II.

Three strains, namely I and I *a*, which formed gas, isolated respectively in February and Sept. 1912, and II, a strain which did not produce gas, which was isolated in Sept. 1912 at the same time as I *a*, were inoculated on 22nd December, 1912 into peptone water tubes containing 20 different carbohydrates, glucosides and alcohols. (See Table III.)

No differences between the three strains were found except as regards the production of gas. Strains I and I *a* however always produced gas when acid was formed, whilst strain II produced only acid and never gas from any of the substances with the possible exception of dextrin, but in all the dextrin tubes small bubbles of gas appeared and the acid reaction was slight even in the case of Strains I and I *a*.

All the strains formed acid and clot in milk, produced indole in broth, and grew on gelatine at room temperature without causing liquefaction.

At the same time three intermediate strains, A, B and C, which had been recently isolated (on Dec. 9, 1912), were inoculated into peptone water containing the same 20 test materials. These latter strains proved to be identical with the three strains I, II and I *a* as regards their acid-forming properties, but varied amongst themselves as to gas production.

Strain A formed no gas, B very little gas and C much gas when grown on glucose peptone water immediately after isolation.

Serum tests.

Serum experiments were also made in order to test the identity of the three strains I, II and I *a*. Three rabbits were inoculated with pure cultures of the different strains, and their sera used for agglutination tests and also for testing the absorption of agglutinins by the different strains. The results as seen in Tables IV and V showed no differences of any note between the three strains. No sign of agglutination appeared with normal rabbit's serum in dilutions of $\frac{1}{200}$ to $\frac{1}{12800}$, nor in 0·85 % salt solution. The agglutination was carried out at the room temperature and was complete in 20 hours.

For comparison three latoratory strains of *B. acidi lactici* (Hueppe) were also tested as regards agglutination. With serum prepared from

TABLE IV.

Agglutination.

Serum I	1/400	1/800	1/1600	1/3200	1/6400	1/12800	(Control salt solution)
Bac. I	⧻	⧻	⧻	⧻	⧻	⧺	–
Bac. II	⧻	⧻	⧻	⧻	⧻	⧺	⊥
Bac. I *a*	⧻	⧻	⧻	⧺	+	–	⊥
Ser. I a							
Bac. I	⧻	⧻	⧻	⧻	⧻	⧺	–
Bac. II	⧻	⧻	⧻	⧻	⧻	⧺	–
Bac. I *a*	⧻	⧻	⧻	⧻	⧺	⊥	–
Ser. II							
Bac. I	⧻	⧻	⧻	⧻	⧻	+	–
Bac. II	⧻	⧻	⧻	⧻	⧻	⧺	–
Bac. I *a*	⧻	⧻	⧻	⧻	⧺	⧺	–
Bac. ac. l. 1	–	–	–	–	–	–	–
Bac. ac. l. 2	–	–	–	–	–	–	–
Bac. ac. l. 3	–	–	–	–	–	–	–

⧻ = complete. ⧺ = nearly complete. + = marked. ⊥ = very slight.
– = no agglutination.

TABLE V.

Agglutination after absorption of agglutinins.

Ser. I (*abs. with Bac. I*)	1/800	1/1600	1/3200	1/6400	1/12800
Bac. I	⊥	–	–	–	–
Bac. II	+	+	–	–	–
Bac. I *a*	⊥	⊥	–	–	–
Ser. I (*abs. with Bac. II*)					
Bac. I	+	⊥	–	–	–
Bac. II	+	+	⊥	–	–
Bac. I *a*	⊥	⊥	–	–	–

TABLE VI.

Ser. II (*abs. with Bac. I*)	1/800	1/1600	1/3200	1/6400	1/12800
Bac. I	⧻	⧺	+	⊥	–
Bac. II	⧻	⧻	⧺	–	–
Bac. I *a*	⧺	⧺	+	–	–
Ser. II (*abs. with Bac. II*)					
Bac. I	⧻	⧻	⧺	–	–
Bac. II	⧻	⧻	⧺	⊥	–
Bac. I *a*	⧺	⧺	+	–	–

(Bac. I and I *a*, gas-formers. Bac. II, non-gas-former.)

Bacillus I all three strains agglutinated fairly well in dilution of $\frac{1}{8000}$ and very slightly in $\frac{1}{3200}$. With serum prepared from *Bacillus II* no agglutination occurred in a dilution of $\frac{1}{800}$ nor in higher dilutions.

The agglutination of typical laboratory cultures of *B. acidi lactici* was therefore slight with Serum I and very slight or absent with Serum II. This evidence as far as it goes points to a closer relationship between the three strains I, II and I α than between any of them and the laboratory strains.

The two sera I and II prepared with strains I and II respectively were used for absorption experiments and each serum was absorbed with each of the two bacilli separately. The results given in Tables V and VI show an approximately equal absorption by the different bacilli, of the agglutinins for all three strains.

The complete uniformity of the sugar tests as regards acid production and the strong confirmation of the identity of the three strains given by the agglutination tests leave little room for doubt as to the identity of origin of the varieties which form gas and those which do not.

It appears almost conclusively proved that they all originated from a single strain.

Transformation of one variety into another.

As a further test of the identity of the two varieties, attempts were made by culture to transform one variety into the other.

After many failures it was eventually found possible to transform the non-gas-forming variety into the variety which produced gas from sugars. Thus completing the proof of the essential unity of the two varieties.

Cultures of the two extreme varieties remained constant for over four months when subcultured at irregular intervals on agar or broth. In order to obtain information as to the media which favoured either variety, mixtures of the two forms were made by introducing drops of broth cultures of the two varieties into tubes of broth in different proportions, and subculturing daily after incubation into glucose and lactose peptone water. On subculture from mixed cultures after 18 days incubation gas was produced from lactose but little or none from glucose. When 1 % glucose tubes which had been inoculated with mixtures in broth, were incubated and subcultured daily in glucose and lactose, after incubation for 48 hours, only acid and no gas were produced in the sugars. A similar result was obtained in subcultures after four days, but on the fifth day the subcultures failed to grow.

Glucose tubes containing pure cultures of the gas-former were also sterile after four days incubation. This and similar experiments showed that in glucose cultures the gas-former died out in four days to a week at 37° C., but the non-gas-former lived seven to ten days under the same conditions.

In lactose tubes inoculated with a pure culture of the gas-former, subcultures grew and showed production of acid and gas after at least eight days; and after at least five days in the case of mixed cultures, subcultures gave similar results.

It was found too that cultures of the intermediate varieties which when first isolated formed acid and no gas from glucose but acid and gas from lactose, changed so as to produce acid and gas from both sugars after growth in broth for two or three days, but after growth in glucose peptone water for a similar period sometimes no gas was formed from either sugar.

In accordance with these results attempts were made to procure a variation in the gas-former by plating out after three or four days' growth on glucose peptone water, and examining a number of colonies in expectation that if a non-gas-forming variant occurred it would be encouraged in the acid glucose culture in preference to the gas-former.

No such variant could be demonstrated by these means. Attempts were also made, so far without success, to select out a gas-former from a culture of the variety II by prolonged incubation in weak (0·05 %) glucose broth or in ordinary broth, and also by repeated subcultures in ordinary broth continued for over a month.

Penfold (1911, 1912) found that his chloracetate variant of *B. coli* which produced no gas from glucose readily produced gas from sodium formate, showing that in all probability the peculiarity of this variant consisted in a defect in its power of making formic acid from the sugar.

He found, moreover, that by growing *B. typhosus* and his chloracetate variant together in glucose peptone water gas was formed which he attributed to the breaking down of the formic acid made by the *B. typhosus.*

Harden and Penfold (1912) subsequently confirmed this view by showing that the amount of formic acid produced by the chloracetate variant was much less than that produced by the original strain of *B. coli.*

In order to find out whether my gas-former (II) had similar characteristics as regards gas formation from formates, cultures were made with a loop of a broth culture of variety II (1) together with

B. typhosus in glucose peptone water, (2) in peptone water and in broth containing sodium formate with the following results:—

The gas-forming variety (I) produced abundant gas from peptone water containing sodium formate in 24 hours.

Variety II which did not form gas from sugars or alcohols was shown to be a slow gas-former from sodium formate. (See Table VII.)

Similar results have been obtained in broth containing larger percentages of formate.

TABLE VII.

One loop of broth culture of II (non-gas-former) added to each tube and incubated at 37° C.

Tube		Time	Result
1	1 % glucose peptone water + five drops of a *B. typhosus* culture	7 days	Acid, no gas
2	0·5 % sodium formate in peptone water	5 ,,	No gas
3	1·0 % ,, ,, ,, ,, ,,	5 ,,	Some gas
4	2·0 % ,, ,, ,, ,, ,,	5 ,,	No gas
5	1·0 % ,, ,, ,, broth	2 ,,	Some gas

TABLE VIII.

Cultures of variety II (non-gas-former) in media containing sodium formate subcultured to lactose.

Date of culture	% formate	Date of subculture to lactose	Result in lactose
24/1/13	2 % in peptone water	29/1/13	Acid on 30/1/13
		31/1/13	,, ,, 1/2/13
,,	1 % ,, ,,	29/1/13	,, ,, 30/1/13
		31/1/13	,, ,, 1/2/13
,,	0·5 % ,, ,,	29/1/13	,, ,, 30/1/13
		31/1/13	,, ,, 1/2/13
,,	1 % in broth	29/1/13	Acid and gas on 30/1/13 (small quantity)
		31/1/13	Acid and gas on 1/2/13 (large quantity)

It seemed possible that this late yield of gas was due to a "training" or selection of the bacilli which acted on the formate under suitable conditions of growth, especially in the absence of acid which appears to inhibit or destroy the gas-formers as shown above.

The cultures of II in formate (see Table VIII) were therefore subcultured to lactose peptone water with the following results:—

The 1 % formate broth was also plated out on agar on the 31st Jan. and after incubation showed colonies of different sizes. Five large colonies when inoculated into lactose broth gave acid and no gas as before, but of nine small colonies inoculated into lactose peptone water, eight gave acid and gas after incubation for 24 hours. The gas-forming property still remained after subculture on ordinary nutrient broth.

These experiments appear to show that the defect in variety II associated with its inability to form gas is not a want of power to make formic acid but to an inability to split the formic acid formed under ordinary conditions of culture in glucose peptone water, and that the power to produce gas from formates may be acquired in a neutral solution of sodium formate in broth.

The new variant (II *a*) of variety II was examined by inoculation of the formate broth culture into a series of sugars and alcohols, and the identity of its reactions with those given by variety I was shown.

Further work is being done on these varieties.

Discussion of results.

There is no direct evidence in the case of the bacillus described above as to which variety should be considered the original parent and which the more recent variant. However, the fact that the gas-former corresponds in almost all its characters with the *B. acidi lactici* which is very commonly met with in the faeces, makes it more probable that this is the parent form.

Variation in bacteria which is shown by the loss of some property has been attributed to a general lowering of the functional activity of the bacteria concerned, in which one function has been suppressed before the remainder, because it has been more recently acquired or is less essential to life.

Thus Scheierbeck's variant streptococci were produced by the inhibiting agent—carbolic acid—and the impaired acid-forming function of the bacteria was associated with slower growth.

Such an explanation, if cogent, would perhaps lessen the importance of lost characters from the theoretical standpoint of evolution, though for the practical purpose of diagnosis the loss and the acquisition of a characteristic are equally important.

The loss or impairment of a function may however be associated with a gain in another direction as in the case of Scheierbeck's streptococcus cultures which produced less acid during the first period of growth, but continued to grow for a longer time in the cultures and eventually formed a higher percentage of acid than the more rapid acid-formers.

Penfold's variant on chloracetic-agar which had lost its power to form gas from sugars, grew in larger colonies on the selective medium than the original type, indicating an increased vigour rather than a diminished one.

One of the varieties of the bacillus which is the subject of this paper, had no power of forming gas from sugars or alcohols, and in some instances appeared to grow in smaller colonies on agar than certain races of the gas-forming variety. When however cultures of the two varieties were mixed in broth and the mixture plated out on agar the gas-former grew in smaller colonies than the variety whose power to form gas was in abeyance. This was shown by inoculating a number of the small and of the large colonies into glucose peptone water.

Moreover, the variety which did not form gas survived for a considerably longer period in glucose peptone water than the gas-former.

The suggestion, therefore, that a general weakness is the rule in the case of strains of bacteria which show loss of function in one direction, is by no means supported by the evidence in all cases, and the apparent loss may be fully compensated in other directions.

Some special features of the varieties described above remain to be mentioned.

(1) The power of producing gas from alcohols is absent in the case of those strains which have not this function in regard to sugars. In this respect the anaerogenic strain resembles those produced by Revis by cultivating *B. coli* on malachite-green, and also differs from Penfold's variant in that it does not readily produce gas from formates.

(2) The intermediate strains which I obtained from colonies on the original plates mostly showed the remarkable characteristic that they produced a full amount of gas from lactose in 24 hours, but none or very little from glucose in the same time. This behaviour is difficult to explain since *a priori* it seems probable that the lactose is first split into glucose and galactose before these sugars are further acted on with the production of acid and gas.

The suggestion that the gas was formed from the galactose split off from the lactose appeared to be negatived by the fact that when grown in galactose and in glucose, these strains yielded very small amounts of gas from both these sugars, but from lactose at the same time a large yield was obtained.

This observation however does not stand alone. Penfold (1911, 1912) found that during the selection of his chloracetate varieties, the power to form gas from glucose disappeared before the same function as regards lactose.

Revis also had the same experience. Sörensen too noted a somewhat similar phenomenon, as is shown in Table I.

(3) The relative proportions of the different varieties obtained from the samples of urine varied somewhat on different occasions. (See Table IX.)

Thus of 70 colonies picked off the plates made on the 8th December, 21 formed full gas, and 24 formed acid only, whereas 25 showed intermediate characters.

TABLE IX.

Number of colonies examined on each occasion.

Sample of urine...	R. 1	R. 2	R. 3	R. 4	R. 5	R. 6	R. 7	
Date...	12/9/12	20/9/12	10/10/12	31/10/12	8/12/12	31/12/12	17/1/13	Total
Full gas I	4	3	4	3	21	5	4	44
No gas II	2	1	4	3	24	15	14	63
Intermediate III. (No gas from glucose)	3	0	1	0	0	1	3	8
Intermediate IV. (Some gas from glucose)	3	2	0	0	25	3	9	42
	12	6	9	6	70	24	30	157

Of a total of 157 colonies picked off the original plates on the last seven occasions, 44 produced a large amount of gas from glucose, lactose and mannite; 63 formed no gas from any of these three substances and 50 were intermediate in character. Of these latter eight produced no gas from glucose, while the lactose tubes showed a large amount of gas in the Durham's tubes. The remainder formed very little gas from glucose but a large amount from lactose. In each case the gas production was judged by the gas collected in the small Durham's tubes.

It seems unlikely that so large a proportion as 50 isolated colonies out of 157 (31·8 %) were formed from two bacilli of different varieties. These intermediate forms must therefore be regarded as having arisen from single bacilli of intermediate character or from bacilli which at once gave rise to individuals of varying type.

(4) It has not been found possible to maintain the intermediate strains constant. When plated on agar from broth, or glucose or lactose

peptone water cultures, the colonies when inoculated into sugar media usually conform to one or the other extreme type of variety, but occasionally a culture showing intermediate characters has been obtained after plating out the culture three times in succession.

CONCLUSIONS.

1. A bacillus belonging to the *B. acidi lactici* group has been repeatedly isolated during 11 months from the urine of one patient, and no other Gram-negative bacillus has been found in the same urine during this period.
2. The bacillus has occurred in two varieties which differed as regards gas-formation only. Variety I formed gas from sugars and alcohols and variety II formed acid and no gas from the same sugars and alcohols.
3. The two varieties gave identical serum reactions both as regards agglutination and absorption of agglutinins with specific sera prepared from rabbits immunised with the respective varieties.
4. Intermediate varieties as regards gas production also occurred, but were not constant when subcultured.
5. Varieties I and II remained constant in their characters after four months' subculture on broth and agar.
6. Variety II which at first did not produce gas from sugars was induced to do so by first growing in a solution of sodium formate in broth.

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A COMPARATIVE EXPERIMENTAL STUDY OF THE LEPROSY CULTURES OF CLEGG, DUVAL, KEDROWSKY, ROST, AND WILLIAMS.

Being a Contribution to the Section of Tropical Medicine at the Annual Meeting of the British Medical Association, Liverpool, 1912.

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BEFORE proceeding to explain the specimens being demonstrated I am glad to be able to state that the work carried out during the past twelve months only enables me to confirm and amplify the conclusions of my preliminary communication to this section last year.

The only point I have to modify is that which regards the sporadic cases of leprosy in persons who had never left this country. On careful investigation it was found that in one case where the patient denied having been abroad the statement was untruthful. In another case the father and mother and also the grandmother on the maternal side had been lepers, and the boy who contracted leprosy had lived with them when their disease was already in an advanced stage. It was, therefore, not possible to prove an infection through rats in these cases, though I still consider the transmission of infection from rat to man as theoretically possible.

I should like to make a special point of the fact, if I may, that this is above all a demonstration, and that the paper I am reading is mainly explanatory.

I have endeavoured to substantiate all the statements made in this communication by microscopical sections, the number of which has only been limited by the microscopes at my disposal.

The conclusions I have now arrived at, over and above those set forth in my previous paper, are the following:

1. Histological, serological, and clinical evidence lead us to believe that the organism of leprosy is a slowly multiplying, very slightly toxic bacterium, closely allied to the so-called "tubercle" group, but in so far distinguishable from it by not being artificially cultivable as an acid-fast rod on the ordinary media which are made use of to isolate the tubercle "bacillus," and, moreover, in not being

pathogenic for guinea-pigs to the extent that human, bovine or avian tubercle are.

2. Rapidly growing, more or less acid-proof saprophytes are ubiquitous, so that even the repeated cultivation of such from cases of leprosy by no means proves their relationship to the disease in man. Much further identification is required in order to ascertain their true significance.

3. With the exception of Kedrowsky's, no leprosy culture has been, so far, sufficiently identified to enable the statement to be made that its properties and appearance are identical with those of the morphological entity called Hansen's "bacillus," because only Kedrowsky's and the author's cultures have produced lesions in animals absolutely identical with those found in leprosy of the human being and rats.

4. The author's cultural and animal experiments lead him to believe that Kedrowsky's culture is true leprosy, and that Kedrowsky's views of the varying morphology and staining properties of this organism in artificial cultures are correct.

5. Other cultures appear to belong to the group of acid-proof, chromogenic, rapidly growing saprophytes, such as have been found in butter, milk, grass, smegma, urine, dung, earth, etc. Their relationship to leprosy is not clear.

6. Generalized leprous lesions—that is, metastasis in the internal organs—have been produced in rats by the intratesticular injection of the abdominal glands of a rat which has been injected, also intratesticularly, with the ground-up nodules of a leper.

7. A culture from a case of rat lepra has been so far identified as to enable the assertion to be made that it is a pure culture of the organism causing the disease in rats. This culture appears to the naked eye, in its microscopical morphology and staining reactions, very similar to the strain isolated from a leper.

The histological evidence that leprosy does not only attack the skin, but in advanced cases also causes visceral lesions not at all dissimilar in parts to what is seen in tuberculosis, is a fact well known to those who have had occasion to carry out a sufficient number of autopsies of lepers. A review of the subject has been given by Schaffer and also Kedrowsky, who have shown that under certain circumstances giant cells very similar to those of the Langhans type may occur in lepromata, and that sometimes the resemblance between a leprous affection and tuberculous disease can be so complete that only experiments with guinea-pigs enable the distinction to be made.

Lepers are known to react with tuberculin in a similar fashion to tuberculous patients. For further information on the subject I can but refer to the work of Brinckerhoff and the paper of Babes in *Lepra*, 1910, p. 322. Clinically the disease is similar in many points to tuberculosis because of its long incubation, its slow progress, its intractability by any of the drugs known so far.

The similarity of the morphology and staining properties of Koch's and Hansen's "bacillus" is too well known to require repetition. All these observations lead us to believe that the culture of leprosy, or, more exactly, of Hansen's "bacillus," must belong to the so-called "tubercle" group.

If we consider how "leprous" lesions can be imitated under certain circumstances by avian tubercle when injected into fowls and guinea-pigs, we may expect the culture to be similar to avian tubercle. Such microscopical specimens have been published in the last report of the Tuberculosis Commission.

In any case, as said before, it clearly belongs to the "tubercle" group, which consists of human, bovine, and avian tuberculosis.

Quite a different group of organisms altogether, especially as far as their cultural characteristics and pathogenicity for animals are concerned, is formed by the extremely ubiquitous more or less acid-proof saprophytic bacteria, which are exemplified by the *B. smegmae*, *B. phlei*, the numerous acid-fast rods isolated from butter, milk, grass, nasal discharge, urine, dung, earth, etc., by Rabinowitsch, Lombardo-Pellegrino, Binot, Nabarro, Korn, Grassberger, Marpmann, Moeller, Karlinski, Kersten, and others. To the same group belong no doubt the acid-fast bacteria to be found in tap water, and which I have found to possess definite acid-resisting filamentary branching forms, which break up into acid-fast rods. This organism appears to be the one observed by Brem, Beitzke, Schern, and Dold.

The outstanding feature of this latter group is the capacity for growing easily at room temperature on any of the usual laboratory media such as common agar-agar or gelatine. They are non-pathogenic for guinea-pigs in small doses or if injected alone. If injected intraperitoneally in sufficient quantity into guinea-pigs together with butter or fat they are capable of producing nodules on the peritoneal surface not at all dissimilar to what can be seen after injection of human tubercle, and at times the distinction may present difficulties. If injected intravenously in great quantity they may produce caseating nodules in various internal organs, which under certain circumstances may contain an enormous quantity of acid-fast rods, which possibly are only deposits of the organisms injected.

These organisms are generally easily regained in pure culture from the animals into which they have been injected, but though the growth can be very slow for the first generations, gradually it gets more and more rapid till practically all can be made to show a definite growth after three days, on common agar at 37°. Even a fortnight may elapse before the first colonies appear.

The bacteria of this group frequently produce a brilliant yellow or red pigment of varying intensity according to the media they are grown on. Some are almost constantly milky white, such as *B. smegmae*, Karlinski's "nose-bacillus," Moeller's "grass bacillus," A dry, white growth such as Marpmann's acid-fast organism from urine may turn moist yellow and at other times take on a reddish hue, so that it is difficult to make use of pigmentation or similar features as a means of distinguishing one growth from another.

The whole group, therefore, can be roughly differentiated from the family of "tubercle bacilli" of warm blooded animals, from which we must except Hansen's bacillus, by its not being pathogenic for guinea-pigs in small quantities and in growing easily at room temperature on gelatine.

Having approached the problem of leprosy as an investigator with the definite purpose of comparing the claims of different cultures which had lately been published by various authors, I soon found it necessary to compare the behaviour of this large group of acid-fast organisms with that of human, bovine, and avian tubercle cultures from various sources, any leprosy cultures I succeeded in getting being only considered from the standpoint of confirmatory evidence. Once it had been settled which of the many cultures was really Hansen's "bacillus," I could then proceed to identify my strain. This work has been extremely slow in progressing, and I must acknowledge with gratitude the help that has unstintingly been given me by the Lister Institute and staff, and also by my co-worker, Dr. J. M. Martinez of Santamaria Bogota.

The long incubation of directly inoculated leprosy enables me to demonstrate only the second generation of directly transmitted leprosy in rats. The third generation is still under observation, and I do not wish to kill the rat till several months have elapsed.

The following lepra cultures have been extensively tested on animals experimentally: Kedrowsky's strain from Moscow, Duval's culture from New Orleans, Rost's Indian strain, the author's culture from rat leprosy. I have also Clegg's culture, but unluckily was unable to procure Williams's strain, which, however, is considered to be identical to Rost's culture, and appears to belong to the same group of rapid-growing organisms. From personal communication of the author and from Captain Williams's papers on the subject, I see, however, that it is practically non-pathogenic for animals, and admittedly has so far produced no "leprous" lesions in the animals injected with it.

These cultures have now been injected into monkeys, rabbits, guinea-pigs, rats, mice, fowls, and pigeons. The animals were killed at times varying from twenty-four hours to seven months. In no animal, with the exception of a certain proportion of those that had been injected with Kedrowsky's culture and with the culture from human and rat leprosy, were lesions produced which could fairly be considered similar to those occurring in human or rat lepra. The lesions produced by Duval's and Rost's strain would successfully be imitated by intravenous injection into rabbits, or intraperitoneal injection into rats, of large quantities of saprophytic organisms such as Moeller's dung "bacillus" or Grassberger's acid-fast from milk.

This is certainly an apparently sweeping statement; the proofs of my assertion are, however, under the microscopes. You will see that, whether you inject Kedrowsky's culture, the culture I have isolated from a leper in London, the ground-up leprous nodules from human beings or rats, or the organism isolated from rat lepra, you find the same outstanding features in the resulting lesions —an overwhelming quantity of acid-fast rods, to the extent that the whole field of vision may appear to be one mass of acid-fast ruby-red "bacilli," together with a minimal reactive tissue proliferation, hardly any giant cells similar to the true Langhans type as seen in tuberculous lesions, no caseation necrosis, or sclerosis of the vessels.

These are the typical features of the "leprous lesion," and as such they are described by MacLeod in his "Survey on the Present State of our Knowledge of the Bacteriology and Pathological Anatomy of Leprosy," *Lepra*, 1910, iii, 309.

What are the changes brought about by the injection of Duval's strain into animals? Extensive necrosis, production of giant cells of the Langhans type with a necrotic centre, scarcely any acid-fast rods to be detected—in a few words, all the histological features that can be produced by acid-fast saprophytic germs of the timothy grass group. At no time is Duval's organism identical in morphology with Hansen's "bacillus," though at times it resembles it to a certain extent.

Rost's germ is practically non-pathogenic for animals. To produce lesions you must repeatedly inject big quantities or several culture slopes at a time. The resulting lesions show very few acid-fast rods, not at all morphologically similar to Hansen's "bacillus," some caseation, and a copious round-cell infiltration.

Serologically—that is, by means of complement absorption or by agglutination—both these germs react with leprous serums in the same dilutions as the majority of the saprophytic acid-fast and acid-resisting bacteria.

Rost's germ has not been often cultivated, but it still remains to be explained how Duval succeeded in getting, time after time, from the internal organs of his cases an organism that is clearly only distantly related to leprosy and certainly unable to produce any leprous lesions in animals; at least, in the sections kindly sent me by Duval leprous lesions could not be recognized by authorities on the subject such as Dr. MacLeod in London, Professor Jadassohn of Bern, Professor Marchoux in Paris.

Regarding the differentiation of acid-fast organisms by means of various serological methods, such as agglutination, precipitation, and complement absorption, it is known that Duval considers it impossible to carry out these experiments satisfactorily with the serum of lepers. It is quite true that the serum of many lepers reacts very feebly indeed with any acid-fast organism, including the cultures I believe to be genuine leprosy. It so happened that the serum I tested first was the most efficient from this standpoint; none of the others that I examined later showed such marked reactions. But even here, though the titre in relation to Kedrowsky's strain may have been low it was absolutely negative in relation to Duval and Rost's strains. I have published the results of some of my complement absorption experiments, and can but repeat that my results confirm on the whole those of Much and his co-workers, and do not in any way agree with Duval's agglutination tests, so that in this case, whilst awaiting confirmation from an independent source, I have resolved to repeat my experiments on a bigger scale with a great number of leper serums. This I have not been able to do so far.

I can only state once more that by gradually diluting in constant proportions the serum and antigen, moreover by suitably preparing and titrating the antigens, one can, as is to be expected, differentiate with appropriate serums leprosy from tubercle, but not Duval's or Rost's strain from Moeller's dung "bacillus" or Grassberger's organism from milk.

It may also be noted that Dr. Ledingham, of the Lister Institute, has carried out several tropin content determinations with leper serums linked against Duval's and Kedrowsky's strain of leprosy. Duval's strain was not taken up specifically by the leucocytes employed in the presence of inactivated leper serum, whilst on several occasions a very high tropin content could be shown to exist in the serum of certain cases of leprosy when tested against Kedrowsky's culture.

I have no difficulty in admitting that these experiments require repetition on a larger scale; but in the meanwhile it remains an undeniable fact that leprous lesions have been produced in rats and mice by injection of cultures regained from men and rats affected with lepra, as the specimens shown clearly demonstrate. Moreover, that so far no lesions similar to those occurring in human beings have been produced either by Rost's or Duval's culture, so that definite and conclusive serological or experimental proof must be awaited before considering them as cultures of Hansen's "bacillus."

Turning now to the question of the communicability of leprosy to animals, I have been able to persuade myself that it is possible to transmit the disease directly to rats from the human being by injecting ground-up lepromata into the testes. The incubation is exceedingly long and in many cases the lesions are only localized. Since publishing my last paper on the subject in the BRITISH MEDICAL JOURNAL of February 24th, 1912, I have been able to produce in the second generation definite leprous lesions in the spleen, liver, and lymphatic glands of the rat injected, as you will see in the specimens placed under the microscope for your inspection.

In the first instance the localized lesions took four months to develop; the second rat, injected with a lymphatic gland from the first animal, was killed six months after inoculation. and showed great numbers of acid-fast rods in the viscera, which I am now demonstrating. I have no doubt that a multiplication of the "bacilli," though doubtful in the first instance, did take place in the second generation. Culture attempts were unsuccessful.

In a demonstration held before the Society of Tropical Medicine and Hygiene on February 15th, 1912, I announced with Dr. Martinez the culture of an acid-fast rod from a case of rat leprosy. At the time I was not able to give a description of the fish-juice-agar used in the isolation of the organism, owing to the short time at my disposal. I do so now, remarking, however, that though I consider the medium to be quite good for the isolation and culture of most acid-fast organisms, still I do not believe that it can enable us to guarantee a culture in all cases of rat leprosy. The culture on artificial media is a result depending on some factor connected with a special stage or condition in the developmental cycle of the filamentary, branching organism, which I consider to be the parent form of the "bacillus" of rat leprosy, as I also consider a filamentary, branching, non-acid-fast organism to be the parent form of Hansen's "bacillus."

The fish-juice-agar is prepared in the following fashion: Mackerel, cod, or bream are carefully cut up in a meat-chopper and mixed with an equal quantity of sterile distilled water. This mash is placed to freeze hard in the refrigerating room, and next day rapidly thawed. The liquid is separated from the solid matter by first straining it through a thick cloth, after which it is further cleared by passing through a paper filter, vacuum being used to hasten the process. A Berkefeld and a Doulton filter are linked up tandem fashion, and the resulting liquid is caught in a sterile flask, kept twenty-fours at 37° to detect any contamination, and filled into test tubes with an equal quantity of 4 per cent. glycerine agar, which has been liquefied and then allowed to cool down to 42–45° C. Contamination must be carefully avoided, as the slopes may not be sterilized or heated after mixing. It is necessary to go quickly through all stages of filtration after thawing, as decomposition is very rapid.

The rat leprosy nodules are thickly smeared over a good number of tubes; I have often used as many as fifty at a time.

Though our rat leprosy culture has now been artificially cultivated for months, it still has not adapted itself to artificial conditions of life so as to grow in a shorter space than two or three weeks at the very least, showing, therefore, that it does not belong to the "saprophytic" group. It only thrives so far on the media that grow tubercle, and shows no growth at room temperature or on gelatine. As you see, it is also a milky-white, absolutely non-pigmented moist growth, very similar to avian tubercle, but non-pathogenic for fowls even in big doses, half a slope or more.

To produce lesions in a guinea-pig one has to inject a slope or more; in small quantities, up to ten loopfuls, it appears to be non-pathogenic. Injected into rats it has caused so far the identical glandular and visceral lesions which are caused by the intraperitoneal injection of ground-up nodules of spontaneous rat leprosy. These slides show that the organism has been identified, and I am now proceeding in my attempts to regain this bacterium from the animals infected—an experiment which has not succeeded so far.

Also in this point the similarity with human leprosy is striking,* so that I still hold that the rat disease and the human disease, if not identical, are as closely allied as human and bovine tuberculosis are.

A point in connexion with human and rat leprosy which I have not investigated during the past year is the question of an eventual transmission of the disease through arthropods. So little is known about the bionomics of Hansen's "bacillus" and the "bacillus" of lepra murium, that it is premature, to say the very least, to decide whether the presence of a few acid-fast rods in any arthropod, be it a bug, mosquito, fly, flea, or louse, has any connexion in the spread of leprosy, especially as we do not know, nor have means of proving, whether the acid-fast organism seen under the microscope is really Hansen's "bacillus" or a simple ubiquitous saprophyte, moreover whether it is dead or alive, and capable of multiplying and infecting. It is quite possible that the non-acid-fast stages of the germ of leprosy are the only ones capable of infecting in small numbers.

Regarding treatment I am awaiting the results of the injections with bacterial extract, made on the same lines as Koch's old tuberculin from one of Kedrowsky's cultures. This substance gives a definite, typical rise of temperature within twenty-four hours in the three nodular cases I have injected with 1 c.cm. of the extract. I do not know if it has any therapeutic value; in any case I do not expect it to produce any conclusive results till many years have passed, during which the patients have been under close observation. I believe that we cannot hope for any cure under the time that the disease has taken to develop, which in the majority of cases is a period extending over many years.

To show that I am quite aware of the pitfalls attending therapeutic efforts in connexion with leprosy, I am projecting a series of photographs which I owe to the kindness of Dr. George Turner, showing the remarkable improvement which took place in a young girl leper who had not undergone any treatment whatever during this time. Later on the disease again took a progressive course.

The acid-fast rods found in leprous lesions are so quiescent in the tissues, moreover resist disintegration through the protective substances of the animal body and tissue reactions, that I am unable to expect much from what is called "vaccine" (killed cultures) treatment.

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* Of course this is not the only reason; I have given the others in previous papers.

My conclusions are, in a few words, the following:

1. Only Kedrowsky's culture and similar or identical strains, such as Bayon's, have produced on injection leprous lesions in animals.
2. Duval's and Rost's strains do not cause any leprous lesions in animals. Their cultural behaviour is that of saprophytic organisms. The same applies to Clegg's culture.
3. The direct transmission of leprosy from man to rats has succeeded in the second generation.
4. A culture from a case of rat leprosy has produced on injection into rats identical glandular and visceral lesions to those found in the spontaneous disease.
5. A bacterial extract prepared from the culture isolated from lepers gives a specific rise of temperature, but cases have not been observed long enough to enable any conclusions to be drawn as to its therapeutic value.

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LXII. THE OPSONIC INDEX IN PLAGUE VACCINATION.

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In this paper an attempt is made to correlate the production of immunity in rats vaccinated against plague with the production of immune opsonin in the serum of such animals.

Various antibodies appear in the serum of animals inoculated with plague vaccines, but the agglutinins and bactericidal substances, for example, do not lend themselves to satisfactory quantitative study in the case of plague. The opsonic response has been studied in man by Douglas[1] who found a good response after inoculation with Haffkine's prophylactic. It, therefore, seemed advisable to correlate the development of opsonin with the absolute immunity of some susceptible animal in order to see whether the amount of opsonin produced could be used as evidence of the development of immunity in man.

In most of the following experiments the results obtained with a single dose of vaccine are recorded in relation to the opsonic index: in others the results of repeated dosage are shown. These results should be considered in the light of actual immunity experiments with test lethal doses of plague, as performed on vaccinated animals by S. Rowland and described above (p. 367).

The scope of the enquiry resolved itself into the following points:

1. Is there any correlation between immunity and opsonin-production in plague infection?
2. What is the influence of previous vaccination in the experimental animal?
3. What is the source of opsonin in immune plague serum?

Methods of experiment.

In the following series of experiments the dilution method of Klien (1907) was adopted.

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[1] Communicated to the Pathological Society of London, 1906.

In high concentrations of immune serum phagocytosis of plague bacilli is obscured by (*a*) digestion of the bacilli inside the leucocytes, and (*b*) by clumping of bacilli. By means of the dilution method these difficulties are overcome. The ordinary method of incubating the opsonic mixtures in capillary pipettes did not give satisfactory results as the bacilli were found to precipitate and form clumps during the process, thus giving very uneven counts in smears from the same mixtures. I found the most satisfactory results were obtained by making the opsonic mixtures in small tubes which were subsequently placed in a shaking machine in a 37° C. incubator. The mixtures were incubated for a period of twenty minutes.

Throughout the period of experiment a strain of avirulent plague bacillus (K. 120) was used in the opsonic technique, and in vaccinations with the whole bacillus. The nucleoprotein of the plague bacillus used for vaccination was also prepared from the same source. The cultural characteristics of this bacillus have been given by Rowland (vol. x. p. 563).

The bacterial emulsion was prepared each day from a twenty-four hour agar slope, and throughout the period of experiment agar from the same batch was used. This fact is of some importance as it has been observed that great alterations in the phagocytic index may be caused by using bacilli grown on a fresh batch of agar during the time of experiment (Noon, 1909). The cultures were emulsified in 5 % formalin solution and placed in the hot room (37° C.) for one hour in order to kill the bacilli. This was regarded as a necessary precaution on account of the danger in spreading slides containing live bacilli. In one case a control with live bacilli was made which showed that the employment of formalised cultures did not appreciably alter the phagocytic index. The ordinary methods of obtaining fine emulsions, such as shaking with glass beads, do not give good enough emulsions of plague bacilli for opsonic work. To get over this difficulty the following method was adopted. The killed formalin emulsions were decanted into small centrifuge tubes and spun down for a few minutes. The supernatant fluid was then poured off and the bacterial residue was thoroughly broken up by means of a small sable-hair brush, normal saline solution was added and the process repeated two or three times. Finally a short centrifuging was given to precipitate any remaining clumps, the top layers of the emulsion removed to a separate tube and then thoroughly mixed with a capillary pipette. Emulsions of approximately uniform turbidity were used throughout the experiments.

The pooled serum of ten rats was used for each experiment. The first bleeding was performed before inoculation. The rats were vaccinated subcutaneously in the thigh and were bled from the tails, a couple of drops of blood being taken from each animal. The blood was allowed to clot out over night at room temperature and the serum was separated each morning, and used for opsonic determination the same day. The serum dilutions were prepared immediately before the daily opsonic determinations.

Throughout the experiments I used preparations of my own leucocytes, prepared in the usual manner. The emulsions were used about one hour after preparation in order to give the leucocytes time to recover from any paralysis induced by centrifugalisation, but were not allowed to remain unused long enough to allow any deterioration of activity to set in. One hundred leucocytes were counted on each slide. Broken leucocytes and leucocytes in clumps of more than three were rejected in counting.

In each of the opsonic mixtures one volume of normal saline, one volume of serum dilution, one volume of bacterial emulsion and one volume of leucocytes were used. The counts of the falling dilutions were compared with a saline control in each case and controls of normal rat serum were from time to time employed[1].

Materials used as vaccine.

The specific nucleoprotein employed was made by Dr Rowland in the manner described by him (Rowland, 1908). The powder "B" employed represents the bodies of the bacilli, with their contained nucleoprotein, in a mixture of anhydrous sodium sulphate. The number of grams of the powder used was added to twice the quantity of distilled water at 37° C. and the mixture was then filtered at 37° C. through a Büchner filter. The residue was scraped from the filter-paper and emulsified in distilled water and after centrifuging down a portion of the supernatant fluid was set aside for estimation of nucleoprotein. The nucleoprotein in this portion was precipitated by boiling after the addition of acetic acid and the precipitate carefully collected, washed, dried and weighed in the usual way. The amount of nucleoprotein in the original solution was thus controlled and the necessary dilutions made therefrom.

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[1] With normal rat serum an opsonic effect was found in serum dilutions of [illegible] and [illegible] but never in higher dilutions than these.

When the whole bacillus was employed as a vaccine, the surface growth of six Roux bottles, incubated for five days and killed by heating for half an hour at 60° C., was swept off into 5 c.c. normal saline and thoroughly shaken up with glass beads in a stout flask. Three c.c. of this emulsion were then centrifuged down in a tared centrifuge tube and after several washings with distilled water, the residue was evaporated in a hot-air oven at 105° C. till the weight was constant. The remainder of the emulsion was then diluted down to the required strength. Although plague nucleoprotein is readily reduced in toxic and immunising properties by heating, Rowland has shown that this does not obtain so long as the nucleoprotein remains in the bodies of the bacilli (Rowland, 1911).

The slides containing the opsonic films were fixed with methyl alcohol and stained with dilute Giemsa stain for fifteen minutes.

A. Experiments on rats.

Experiment I.

Each of a series of ten rats was inoculated with $\frac{1}{100}$ mg. of freshly prepared nucleoprotein solution in 1 c.c. normal saline. The animals were bled before inoculation, ten hours later and subsequently every morning. The following table gives the results of opsonic counts from day to day.

Serum dilution	1 Before inoculation	2 10 hours	3 22 hours	4 2 days	5 4 days	6 5 days	7 18 days
1/16	2·53	—	—	—	—	—	—
1/32	1·10	—	—	—	—	—	—
1/64	1·05	—	—	—	—	—	—
1/128	—	—	—	—	—	—	2·05
1/256	—	2·52	—	—	2·10	1·00	1·70
1/512	—	1·96	1·66	—	2·00	1·62	0·98
1/1024		1·80	1·58	1·50	1·02	1·49	0·91
1/2048	—	1·80	1·46	1·37	0·92	1·07	—
1/4096	—	1·00	1·73	·87	0 93	1·01	—
1/8192	—	0·98	1·10	·87	—	—	—
1/16384	—	—	1·02	—	—	—	—
1/32768	—	—	1·02	—	—	—	—
Normal saline control	1	1	1	1	1	1	1

Taking the highest dilution of serum which produces an effect greater than that of normal saline, the following result is obtained.

Before inoculation	10 hours	22 hours	2 days	4 days	5 days	18 days
1/16	1/2048	1/4096	1/2048	1/512	1/1024	1/256

The curve obtained by plotting out these figures is shown on chart I.

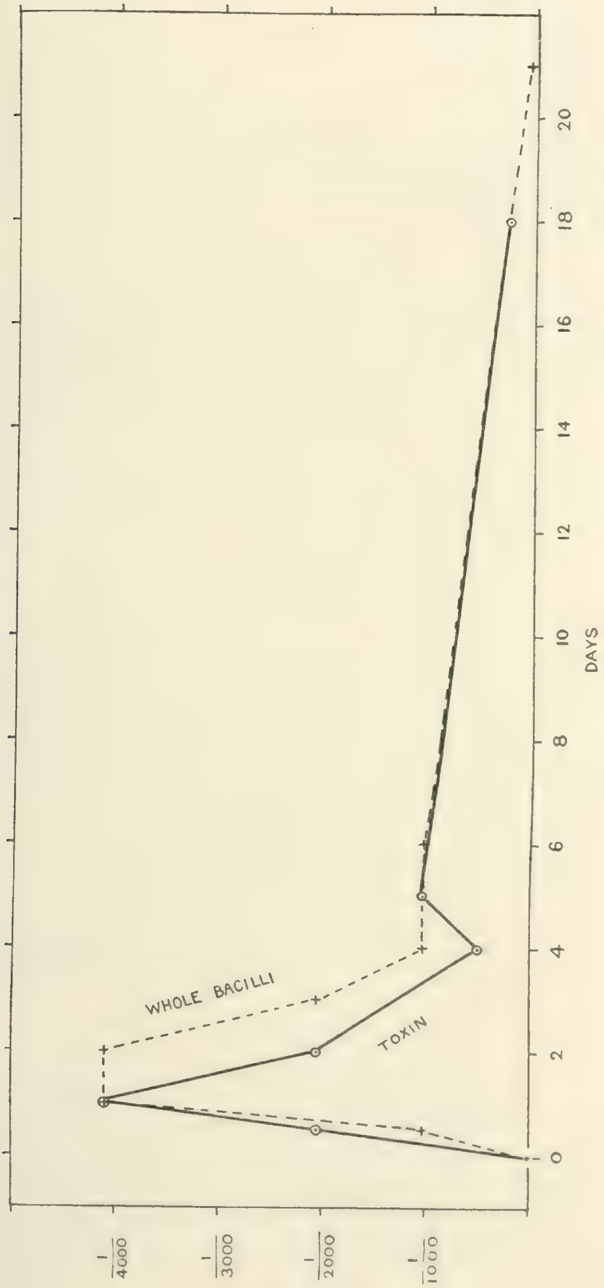
WHOLE BACILLI
TOXIN
DAYS
0
2
4
6
8
10
12
14
16
18
20
1/4000
1/3000
1/2000
1/1000

Fig. 1.

It will be seen from this that the maximum is obtained within 24 hours and afterwards the curve steadily falls so that on the eighteenth day a serum dilution of $\frac{1}{512}$ corresponds to the saline control. It has been shown that full immunity to a lethal dose of virulent plague is obtained within the same period, thus showing that, for this dose of vaccine, the production of opsonin follows the immunity reaction, at least in the early period of immunity.

Experiment II.

Ten rats were inoculated with $\frac{1}{10}$ mg. of whole bacillus vaccine. Rowland has shown that the nucleoprotein content of the bacillus equals about $\frac{1}{10}$ by weight of the whole bacillus, consequently this dose of whole vaccine is comparable to the dose of derived vaccine in experiment I.

The following results were obtained in this series, and the plotted curve obtained therefrom is also shown on chart I.

Before inoculation	10 hours	22 hours	2 days	3 days	4 days	6 days	21 days
1/16	1/1024	1/4096	1/4096	1/2048	1/1024	1/1024	1/64

The results obtained are similar to those obtained in experiment I. The optimum of the curve is displaced slightly towards the right. This difference is so slight that one would not be inclined to place too much significance on the fact, which, however, might possibly be due to the fact that the nucleoprotein in the second case takes an appreciable time to soak out of the bodies of the bacilli.

The general conclusions from these experiments would seem to show, as found by S. Rowland by directly determining the protection aroused in rats to a subsequent fatal dose of living plague, that the soluble nucleoprotein contained in the plague bacillus is the responsible agent in the production of opsonin.

Experiment III.

Ten rats were each inoculated with a very small dose of nucleoprotein solution, *i.e.* $\frac{1}{12000}$ mg. in 1 c.c. normal saline. At the end of ten hours samples of blood were taken but no opsonic response was observed. The curve commenced to rise the next morning and reached its maximum on the third day after which it gradually fell.

Before inoculation	10 hours	22 hours	2 days	3 days	4 days	5 days	6 days	8 days
		1/512	1/1024	1/2048	1/2048	1/1024	1/828	1/512

Compared with the curves obtained with the larger doses in experiments I and II, it will be observed that with this extremely small quantity the maximum is postponed to the right and that the curve does not rise to anything like the same height.

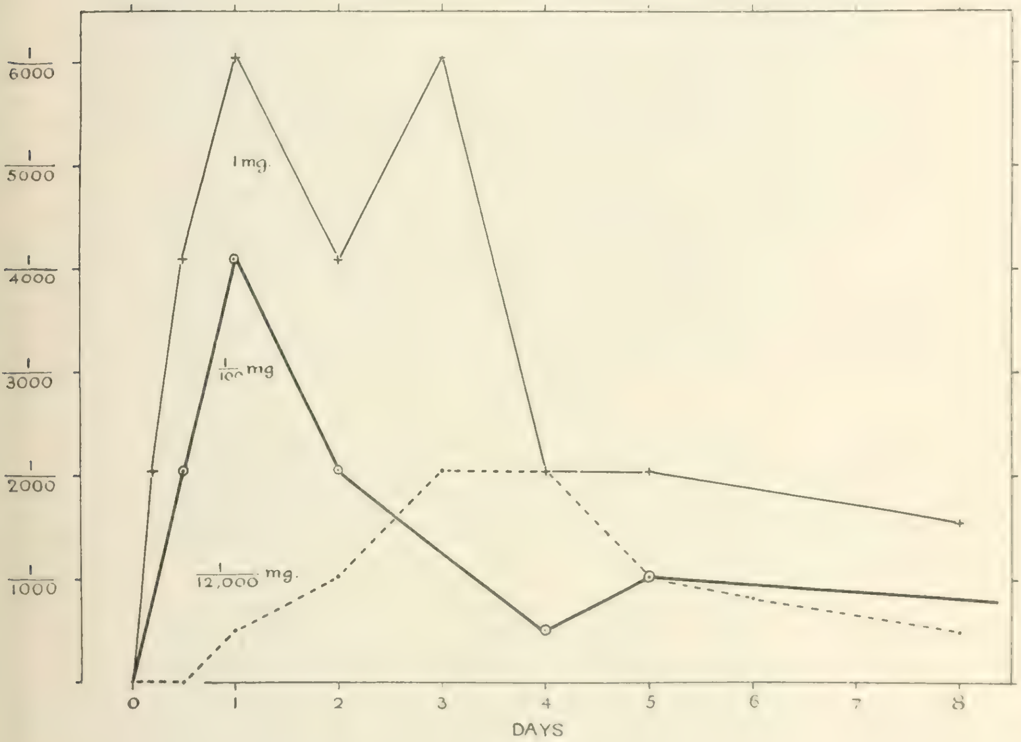

Fig. 2.

Experiment IV.

The ten rats in this series were given a very large dose of nucleoprotein, *i.e.* 1 mg. in 1 c.c. saline. In fresh solution this dose is usually fatal to rats, so the solution was incubated for 48 hours at 37° C. under toluol in order to allow a certain amount of hydrolysis to take place, which Rowland has shown reduces toxicity without interfering with the protective value of the nucleoprotein. In spite of this preliminary hydrolysis, four rats died during the experiment with symptoms of acute plague toxaemia.

The rats were bled 1 hr., 5 hrs., 10 hrs., 22 hrs., and subsequently every morning, after inoculation.

Before inoculation	1 hour	5 hours	10 hours	22 hours	2 days	3 days	4 days	5 days	8 days
—	—	1/2048	1/4096	1/6044	1/4096	1/6044	1/2048	1/2048	1/1536

The curve obtained in this experiment is higher than in the foregoing series, but this increase is not commensurate with the increased dose of vaccine given to the animals. A point has apparently been reached when increased dosage of vaccine does not bring about a proportional increase in opsonin production, as the amount of opsonin present is not much greater than with a tenth of the dose, as given in experiment I.

The drop in the curve after the sixth bleeding is remarkable and may be attributed possibly to the fact that two of the rats bled at this time died shortly afterwards of toxaemia. The curve rose after this, and the general tendency of the curve is towards a maintenance of the maximum for a longer period than in series I and II.

Experiment V.

Series II, which received a dose of $\frac{1}{10}$ mg. of whole vaccine, was given a repeated dose of the same quantity ($\frac{1}{10}$ mg.) thirty-five days later, when the index had been normal for some weeks. The following results were obtained.

Before inoculation	10 hours	1 day	2 days	3 days	4 days	7 days
—	1/4096	1/16384	1/24476	1/24476	1/16384	1/4096

It will be observed that in comparison with the curves obtained in series II, that the ordinates of the curve are very much higher than those obtained in the latter series, also that the optimum point is postponed to the right, taking place between the second and third days instead of between the first and second days.

Rats vaccinated with this dose retain their immunity for about a year. The opsonin production falls in the first week after inoculation, but the animals are still quite sensitive to a fresh dose of vaccine, and greatly increased response is elicited on re-vaccination.

Experiment VI.

In this experiment the washed bodies of the bacilli were employed for inoculation. The nucleoprotein was extracted in the usual way and the residue was washed and centrifuged down daily. This suspension of bacterial bodies was kept under toluol to prevent decomposition. At the end of five days the supernatant fluid was quite free from precipitable nucleoprotein.

Each of a series of ten rats was inoculated with 0·9 mg. of these extracted bodies corresponding to a dose of 0·1 mg. whole vaccine and to 0·01 mg. of nucleoprotein. The serum of these animals was tested from day to day, but no increase in opsonic index was obtained throughout the experiment.

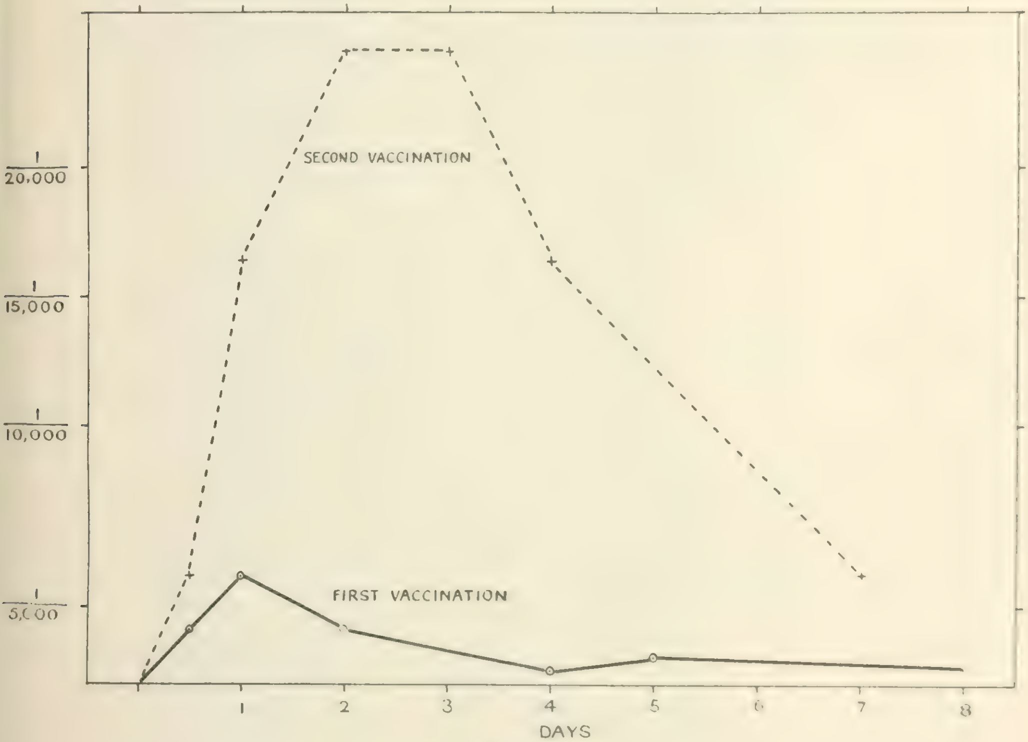

Fig. 3.

Summary of rat experiments.

From the study of the foregoing experiments the following facts appear to emerge. The production of opsonin in immune plague serum at first rises with the production of immunity, and soon returns again to normal leaving the resistance to infection (as ascertained by the direct test) still very high (see fig. 4). A second vaccination shows however that the capacity for response is much enhanced. In the

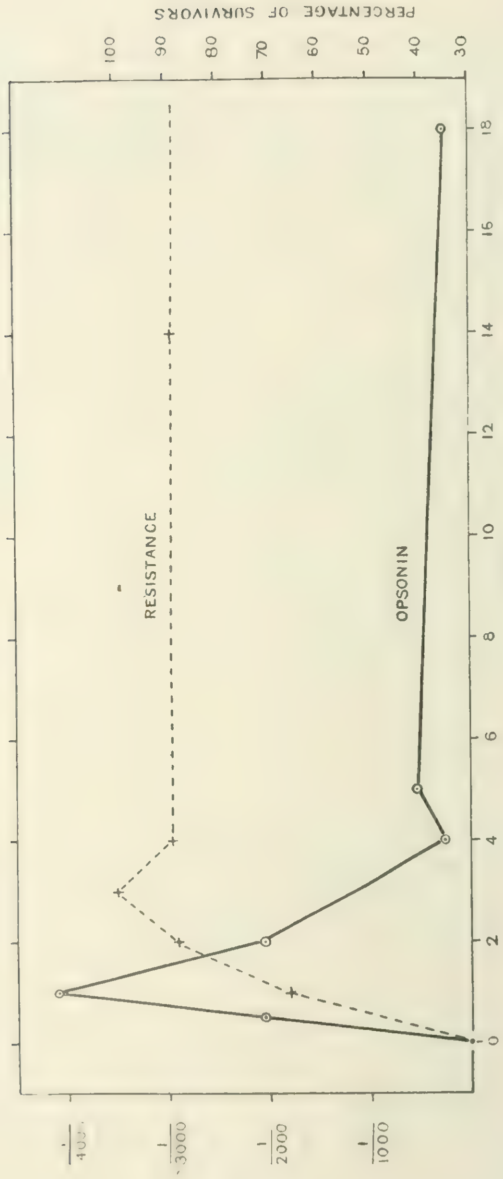
PERCENTAGE OF SURVIVORS
100
90
80
70
60
50
40
30
RESISTANCE
OPSONIN
DAYS
0
2
4
6
8
10
12
14
16
18
1/400
1/3000
1/2000
1/1000

Fig. 4.

curves representing experiments I and II the maxima of the curves are reached during the first or second day by which time full immunity is established. In the case of the very small dose ($\frac{1}{12000}$ mg.) in experiment III the maximum is postponed till the third day, and with the large dose (1 mg.) in experiment IV, the maximum is maintained for a larger period. The relative quantity of opsonin present is greater with the larger dose of vaccine used for inoculation.

In plague vaccination the active principle producing opsonic rise in the serum of rats resides in the nucleoprotein contained in the bacillus. This is shown (1) by the fact that the curves obtained by use of nucleoprotein solution and whole bacillus respectively are practically the same both as regards date of maxima and height of curve, and (2) by the fact that the washed bodies of the bacilli from which all the nucleoprotein has been extracted have no opsonin-producing value.

B. HUMAN EXPERIMENTS.

Experiment I.

I received $\frac{1}{10}$ mg. nucleoprotein in 1 c.c. saline, which nucleoprotein had been hydrolysing under toluol for five days[1]. This solution was first tested on rats with the following results:

2 rats given ·2 mg. each alive after 1 week.
,, ,, ·4 ,, ,, ,,
,, ,, ·6 ,, ,, ,,
,, ,, 1·0 ,, died in one and three days respectively with post-mortem evidence of plague toxaemia.

The arm at the seat of inoculation was somewhat stiff and painful for 48 hours. I suffered from a slight headache next morning but pulse and temperature were normal throughout.

The following results were obtained:

24 hours	2 days	3 days	4 days	5 days	6 days	7 days	9 days
—	—	1/1024	1/768	1/1024	1/1536	1/512	1/512

It will be observed that the maximum point of the curve in this case is very much later than in any of the curves obtained with rats, being on the sixth day instead of the first to third days.

Experiment II.

The pooled serum of five persons who had been at some time previously inoculated with Haffkine's prophylactic and who had been given an inoculation of nucleoprotein solution by me was tested on eight successive days for opsonin after inoculation. The nucleoprotein solution was the same as used in the last experiment, but had been hydrolysing for 12 days.

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[1] This dose of nucleoprotein represents the amount of nucleoprotein contained in 1 c.c. of Haffkine's prophylactic.

Initials	Symptoms	Last dose of Haffkine's prophylactic
J. W.	Slight stiffness in arm and dizziness next morning, pulse and temperature normal.	About 12 months
C. C.	Slight stiffness in arm next morning, pulse and temperature normal.	,,
S. R.	No symptoms, pulse and temperature normal.	Over 12 months.
I. R.	Slight stiffness in arm, pulse and temperature normal.	10 months.
F. W.	No symptoms, pulse and temperature normal.	About 8 months.

The following results were obtained with the pooled serum:

1 day	2 days	3 days	4 days	5 days	6 days	7 days	8 days	9 days
—	—	1/512	1/512	1/1024	1/1024	1/512	1/512	1/256

It will be observed that the top of the curve is reached between the fifth and sixth days after which it gradually falls. No increase of opsonin was observed until the third day. The five persons inoculated were practically without symptoms throughout the experiment.

Experiment III.

The pooled serum of five persons who had never been previously inoculated with plague vaccine and who had been given a dose of $\frac{1}{10}$ mg. hydrolysed nucleoprotein, subcutaneously, were tested for opsonin. The nucleoprotein solution had been hydrolysing for eleven days. A dose of 1 mg. was non-lethal for rats.

Initials	Symptoms
H. D.	Slight erythema at seat of inoculation, pulse and temperature normal.
H. P.	,, ,, ,, ,, ,,
A. S.	No symptoms.
C. F.	,,
G. M.	,,

Before inoculation	3 days	4 days	5 days	6 days	7 days	10 days
Less than 1/32	1/128	1/128	1/256	1/384	1/256	1/192

In this series the opsonic response was not as great as in series II. The number of persons in each series is small and one would hesitate to draw any definite conclusion from these results, but as far as they go they tend to show that pre-existing sensibility has an influence on the opsonic index in human plague vaccination.

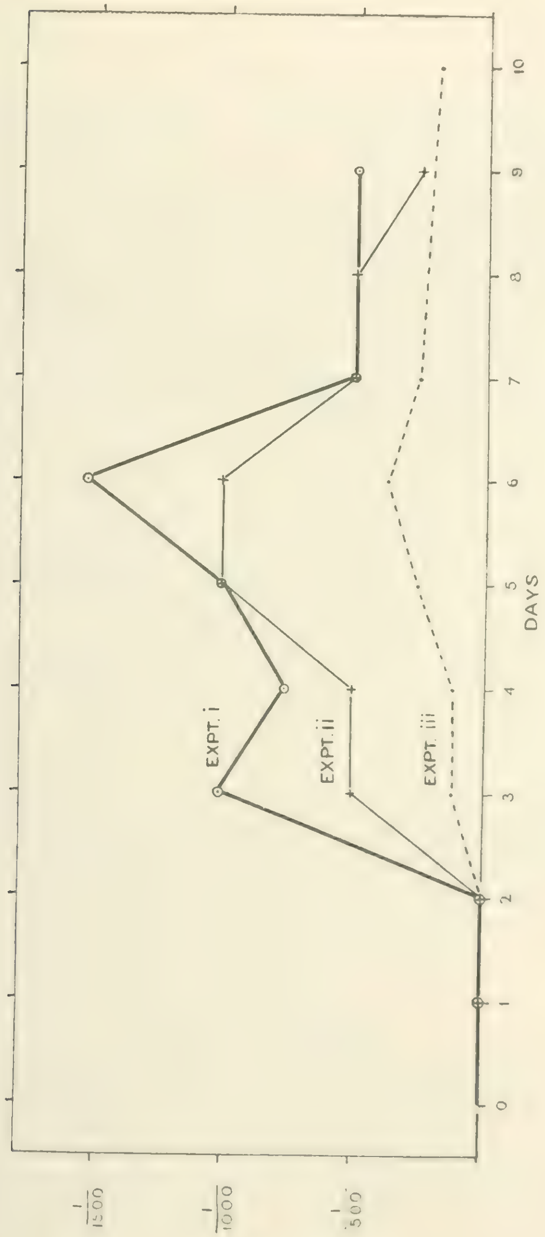

Fig. 5.

CONCLUSIONS.

1. The substance which produces a rise in the opsonic index in immune plague serum is the nucleoprotein contained in the bodies of the bacilli.

2. The washed bodies of the bacilli when used as a vaccine do not cause any increase in the opsonic index.

3. Within limits, the larger the dose, the greater the opsonic response.

4. A second dose of vaccine raises the index above the maximum of the first inoculation, even after the curve has fallen to its normal level.

5. In the early stages, in rats, the opsonic response and the degree of protection aroused rise together.

6. In human beings the maximum response is observed to be much later than in the case of laboratory rats, and the response in a series of previously vaccinated persons is somewhat greater than in a series of persons not previously vaccinated.

7. The local and constitutional effects of nucleoprotein vaccination compare very favourably with those observed in other methods of protective inoculation.

I desire to express my thanks to Prof. C. J. Martin, F.R.S., Director of the Lister Institute, and to Dr J. C. G. Ledingham, Chief Bacteriologist, Lister Institute, for valuable advice and suggestions throughout the inquiry, and particularly to Dr Sydney Rowland, without whose constant advice and help the experiments could not have been carried out.

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THE BACTERICIDAL PROPERTIES OF BLOOD SERUM.

I. THE REACTION-VELOCITY OF THE GERMICIDAL ACTION OF NORMAL RABBIT-SERUM ON *B. COLI COMMUNE* AND THE INFLUENCE OF TEMPERATURE THEREON.

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(With 4 charts.)

THE following experiments, which form part of an attempt to investigate the bactericidal properties of serum, are concerned with the process by means of which certain bacteria are killed *in vitro* when immersed in normal sera. Special attention was paid to the temperature relations of this action because it was hoped that some information might be gleaned which might throw light upon the significance of fever as a reaction to infection.

The method of study was in the main similar to that previously employed by me in investigating the germicidal action of other agents (1908 and 1910) and consists in observation of the time relations of the process. As far as I am aware such a method has not hitherto been applied to the study of the bactericidal properties of serum and by its means data have been obtained which are quite new in character. Under these circumstances it does not seem necessary to give any review of the voluminous literature dealing with other aspects of this subject, but such as bears any direct reference to the matter of this paper will be alluded to as occasion occurs.

Materials and method of experiment.

The normal serum used in the following experiments was allowed to remain on the blood clot for about 18 hours, and then separated from any stray red corpuscles by centrifuging. A small quantity, 2·5 c.c., was measured out into a test tube placed in a thermostat at the required temperature of experiment. In order to test the serum for sterility, sterile broth was added to the small deposit left in the centrifuge tube and the whole incubated at 37° C. for two or three days. No difficulty was experienced in keeping the serum sterile.

The bacteria for the experiment were obtained from a 24 hours' culture in broth at 37° C.; this was filtered through paper to get rid of any agglutinated masses and diluted as required (from 50 to 250-fold) with distilled water. A small quantity of the diluted culture, measured by means of special capillary pipettes each delivering a standard drop equal to 0·02 c.c., was added to the serum tube in the thermostat and the time noted. The serum tube was also fitted with a standard capillary pipette by means of which samples could be withdrawn after successive intervals of time and a definite number of drops taken for plate cultures. Duplicate plates were poured at each time of sampling, necessitating the help of at least one assistant. Gelatine plates were usually employed and they were counted after three to four days' incubation at 20° C.

A more detailed account of the method, including the expedients adopted for maintaining a culture of constant resistance, is given in the previous paper already referred to (1908, pp. 96 and 118).

The experiments set forth in Tables I–X (with the exception of Table IV which is concerned with the action of normal goat-serum upon *B. typhosus*) were all made with normal rabbit-serum and *B. coli commune*.

Table I contains the results of three experiments carried out in duplicate at three different temperatures, viz. 20·2° C., 30° C. and 40° C. The general course of events is more readily comprehended by a glance at Fig. 1, where concentration of surviving bacteria is plotted against time.

After the addition of the bacteria to the serum an interesting series of phenomena takes place which, in the case of all three experiments, can be grouped into three distinct phases.

1. *A latent period*, lasting from 1–4½ hours according to the temperature, in which the number of living bacteria remains stationary or may decrease.

2. *A period of multiplication*, the length of which is also inversely related to temperature, during which the number of bacteria is increased until the concentration is about doubled.

The total lag caused by periods 1 and 2 varied from about 2½ hours at 40° C. to about 4½ and 7 hours at 30° C. and 20·2° C. respectively. This may account for the conclusion of Buxton (1905) that normal rabbit-serum was without bactericidal effect on *B. coli*.

3. *A bactericidal period*, during which the bactericidal action of the serum begins to be manifested and the bacteria are submitted to a slow process of disinfection at a rate depending on the temperature.

TABLE I.

Action of normal rabbit-serum upon B. coli commune.

Exp. 30. I. 12	Temperature °C.	Time, hours	Amount of sample taken, no. of drops	Numbers counted on plates		Mean no. of surviving bacteria in 1 drop serum
I	20·2	0·08	1	2220	1478	1850
		0·5	1	1564	1504	1530
		1·5	1	1100	1084	1090
		3·0	1	1158	1284	1220
		4·5	1	1150	1090	1120
		5·9	1	1416	1772	1590
		6·9	1	2368	2372	2370
		13·1	1	1332	1344	1340
		24·2	3 5	2004 6400*		1050
II	30	0·08	1	1696	1644	1670
		0·5	1	1240	1388	1310
		1·5	1	1268	1284	1280
		3·0	1	2520	2448	2500
		4·5	1	2684	—	2680
		5·9	1	276	289	282
		6·9	1	59	56	57
		13·0	5 1	231 45		46
		24·2	10	20,000*		2000
III	40	0·02	1	2088		2090
		0·05	1	1500		1500
		0·13	1	1296	1362	1330
		0·38	1	1150		1150
		0·87	2	2230		1120
		1·2	2	2576		1290
		1·5	2	4448		2220
		2·5	2	5760		2880
		3·6	3	726		242
		9·6	5	17		3·4
		21·0	10	? 1		0·0

* Approximately.

In Experiment II, at 30° C., there is also evidence of the existence of a fourth stage. The disinfection occurring in the third stage was not quite complete and the few surviving bacteria began to multiply again. This phenomenon was not noticed in Experiment III at 40° C. and Exp. I at 20·2° C. was not complete enough to reveal it.

The sequence of the first three stages set out above was found to be a constant characteristic of the action of normal rabbit-serum upon

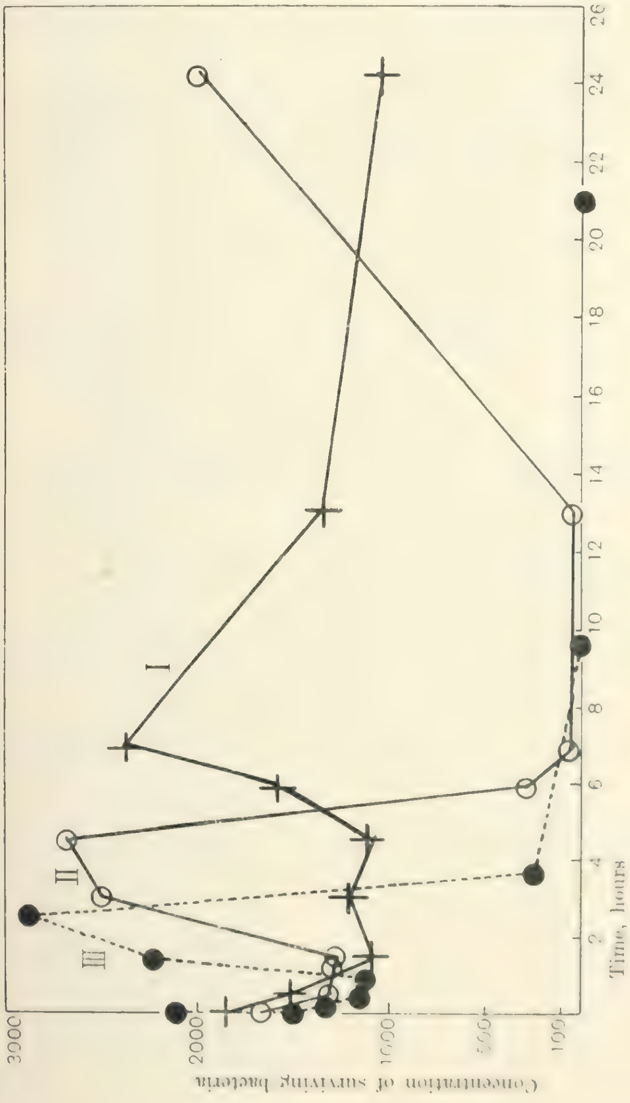

Fig. 1. Action of normal rabbit-serum upon *B. coli commune*, effect of temperature, see Table I.

I. at 20·2° C. II. at 30° C. III. at 40° C.

TABLE II.

Action of normal rabbit-serum (II) *upon* B. coli commune.

Exp. 26. III. 12	Temperature °C.	Time, hours $= t$	Amount of sample taken, no. of drops	Numbers counted on plates		Mean no. of surviving bacteria in 1 drop serum	$\text{Log}_{10} N$	$K=\frac{1}{t_n-t_0}\log\frac{N_0}{N_n}$	Growth constant $A=\frac{1}{t_n-t_0}\log\frac{N_n}{N_0}$	Generation-time, hours $\frac{\log 2}{A}$
I	20	0·08	1	387	378	382				
		1·1	1	327	285	306				
		2·5	1	287	331	309				
		3·5	1	235	253	244				
		4·5	1	267	269	268				
		5·25	1	267	271	269				
		6·3	1	375	348	361	2·557		$(N_0=361;\ t_0=6\cdot3)$	
		7·3	1	450	437	443	2·646		·089	
		8·7	1	555	474	514	2·711	$(N_0=514;\ t_0=8\cdot7)$	·064	
		10·7	1 2	264 538		267	2·426	·142	·076 = mean	3·96
		12·5	1 3	171 420		148	2·170	·142		
		22·0	1 10	9 166		15·9	1·201	·114		
		25·0	1 10	1 107		9·8	0·991	·105		
								·126 = mean		
II	30·1	0·08	1	454	417	435				
		0·5	1	325	343	334				
		1·1	1	286	274	280				
		1·65	1	312	317	314				
		2·5	1	384	340	362				
		3·0	1	499	452	475	2·677		$(N_0=475;\ t_0=3\cdot0)$	
		3·5	1 2	525 1116		547	2·738	$(N_0=547;\ t_0=3\cdot5)$	·122	2·47
		4·0	1 3	379 1128		377	2·576	·32		
		4·5	1 3	179 426		151	2·179	·56		
		5·2	2	170	179	87·2	1·940	·47		
		5·8	2	106	106	53	1·724	·44		

		6·3	2	56		32	1·505	·44		
			5	167						
		7·35	5	81	66	14·7	1·167	·41		
		8·7	5	36	40	7·6	0·881	·36		
		10·7	5	13	9	2·2	0·342	·33		
		12·5	1	1		0				
			10	0				·42 = mean		
		22·0	1	0		0				
			10	0						
		25·0	1	0		0				
			10	0						
III	40·3	0·08	1	325	351	338				
		0·52	1	186	177	181				
		1·1	1	223	255	239				
		1·7	1	346	375	360	2·557		($N_0=360$; $t_0=1·7$)	
		2·4	1	604	—	604	2·781	($N_0=604$; $t_0=2·4$)	·32	0·94
		3·0	1	267	268	267	2·427	—[†]		
		3·5	1	56		45	1·653	1·03		
			3	124						
		4·0	1	13		12	1·079	1·06		
			5	59						
		4·5	2	9		4·2	0·623	1·03		
			5	20						
		5·2	3	1	4	1·0	0·00	0·99		
		5·8	5	Not more than 1 or 2 organisms per plate						
		6·3	5					1·03 = mean		
		7·3	5							
		8·7	5							
		10·7	1	13		14·2	1·152	($N_0=14·2$; $t_0=10·7$)		
			10	143						
		12·4	1	0		0·36	−0·444	0·94		
			10	4						
		22·0	1	0		0				
			10	1						
		25·0	1	4300		4300				
			10	x						

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[*] Briggs' logarithms are used in calculating the value of K in this and all subsequent tables.

[†] Value obtained inconsistent with the others.

B. coli commune. In about an equal number of cases the third stage remained final or was succeeded by a period of further bacterial growth. It is not clear upon what circumstances this depends; the matter is further discussed below, p. 421, where some suggested explanations are put forward.

The quantitative data of the experiments in Table I were not complete enough for satisfactory analysis, such as might throw light upon the nature of the bactericidal action or the effect of temperature thereon. Accordingly a similar set of experiments was undertaken in which determinations of surviving bacteria were made with much greater frequency. The results are set forth in Table II and present a scheme similar to that deduced from the results of Table I, notwithstanding the fact that the serum was obtained from a different rabbit. The first three stages described above are present in all three experiments. In Exp. I at 20° C. the third or bactericidal stage was not completed by the 25th hour, and in Exp. II at 30·1° C. there is no evidence that this phase is other than final. It must be remembered, however, that the march of events in Exp. II is very slow, compared for example with Exp. III at 40·3° C., and it is possible for the final result to remain undiscovered if, as in this instance, the experiment is not continued beyond 24 hours.

In Exp. III, the third or bactericidal stage is succeeded by further multiplication[1] at the tenth hour. Towards the end of the experiment, from the 10th to the 25th hour, this period of growth is followed by a second phase of bacterial death which itself gives place to another period of multiplication. The latter stages in the action of serum upon bacteria need further investigation and for this purpose it will be necessary to arrange experiments of even longer duration than those described in the present communication.

The first three stages of the process were, however, studied with some care in the experiments in Table II and the results obtained may be summarised as follows:

The first or latent period lasted for about 0·5, 1·0 and 5 hours at 20°, 30·1° and 40·3° C. respectively. This stage seems to be merely an instance of the phenomenon which is usually observed when living bacteria are transferred to fresh media and for which no entirely satisfactory explanation has been advanced. For a variable length of time, depending on the conditions of experiment, including the previous history of the seed material and the composition and temperature of the new medium, the number of living organisms remains almost stationary or may even decrease slightly [see Müller (1895); Rahn (1900); Hehewerth (1901); Barber (1908); Lane-Claypon (1909)]. In the present case the initial reduction in numbers, when present is usually much too small to be explained by any hypothesis of agglutination.

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[1] A similar phenomenon was shown by Trommsdorf (1901) in the action of rabbit- and dog-serum upon *B. typhosus*.

The determinations made during the second or growth period were too infrequent for study and this stage was studied separately later on.

During the third or bactericidal period, however, frequent observations were made in order that the process might be investigated in greater detail. It was found that the action proceeded logarithmically, in other words death-rate of the surviving bacteria remained proportional to the concentration of surviving bacteria thus:

$$-\frac{dN}{dt} = KN,$$

where K is a constant and N is equal to the number of bacteria surviving in unit volume. In the seventh column of Table II are given the values of the velocity constant

$$K = \frac{1}{t_n - t_0} \log \frac{N_0}{N_n},$$

where N_0 and N_n are equal to the number of bacteria surviving in one standard drop of serum at times t_0 and t_n respectively. It will be seen that very fair constancy is maintained in the value of K. The same relationship is expressed graphically in Fig. 2 where logarithms of concentration of surviving bacteria as ordinates are plotted against times as abscissae and the experimental points are found to lie upon approximately straight lines.

The fact that the germicidal action proceeds as a "uni-molecular" reaction would suggest that the bactericidal agent was not being exhausted, but was remaining approximately constant in concentration as long as the above relation was maintained. A departure was, however, frequently noticed towards the end of the bactericidal period. At the same time the concentration of living bacteria became so small that it was very difficult to make an accurate evaluation by means of plate cultures. It is possible, however, that a decrease in the value of K such as occurs, for example, in Exp. I might be due to a falling off in concentration of the bactericidal substance or that a few specially resistant forms were being bred which are able to withstand its influence (see

Trommsdorf, 1901). The time occupied for even the most rapid of the experiments was long enough for such a phenomenon to take place and the suggestion receives some support from the fact that a growth period frequently succeeded that of bacterial death.

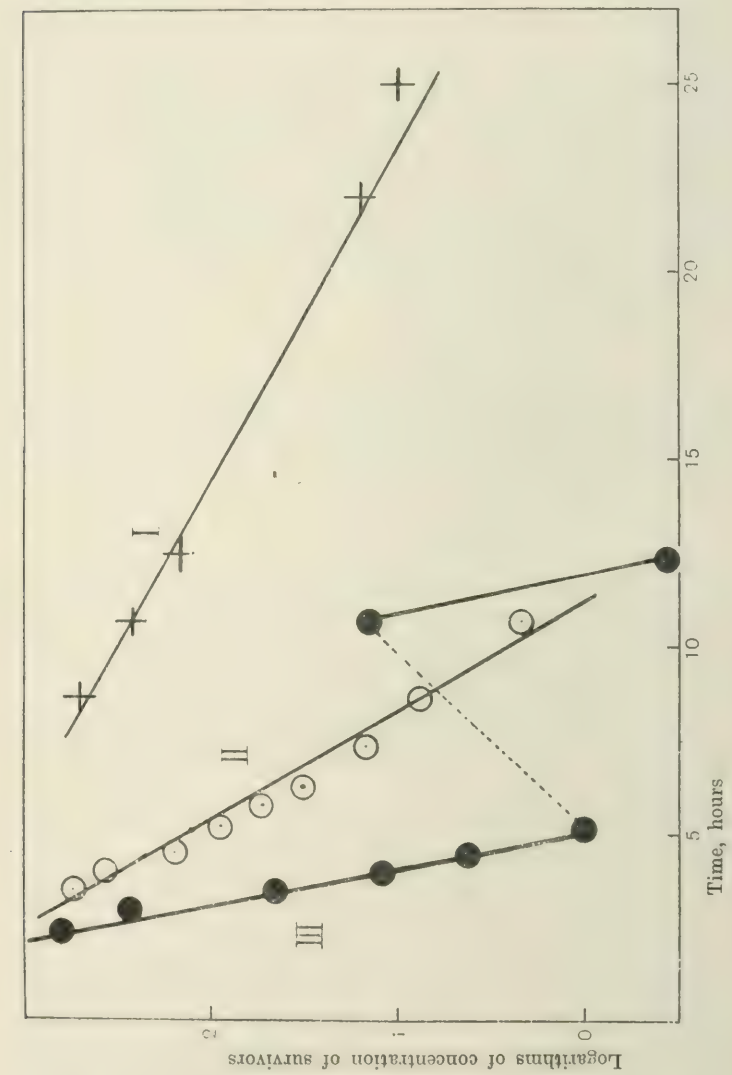

Fig. 2. Influence of temperature upon the germicidal action of normal rabbit-serum on *B. coli commune*, see Table II.

I. at 20° C. II. at 30·1° C. III. at 40·3° C.

The bactericidal action of normal serum thus falls into line with other types of disinfection. In all the cases which have been investigated the process has been shown to take place in accordance with the "unimolecular law," so that the reaction velocity at any time is proportional to the concentration of surviving bacteria. The cases studied include disinfection both of vegetative forms and spores by metallic salts, phenol and other coal-tar derivatives (Chick, 1908); hot water (Madsen and Nyman, 1907; Chick, 1910): sunlight (Clark and Gage, 1903 and Chick, 1910, p. 280); drying (Paul, 1909); the radium emanation (Chambers and Russ, 1912).

Influence of temperature.

The influence of temperature upon the bactericidal action was estimated by a comparison of velocity constants in the different experiments. These are collected in Table III, and it is seen that the effect of temperature is fairly consistent and a logarithmic relationship with reaction velocity is approximately maintained between the temperatures of 20° and 40·3° C., with a temperature coefficient of about 2·8 per rise of 10° C.

TABLE III.

Temperature coefficient of the bactericidal action of normal rabbit-serum (II) *upon* B. coli commune (*from Table II*).

	Temperature C.	Value of the velocity constant, K	$\log_{10}$ $(K \times 10)$	$\log_{10}$ (temperature coefficient per 1° C.)	Temperature coefficient per 1° C.	Temperature coefficient per 10° C.
Exp. I	20	·126	·100			
				·0518	1·13	3·30
Exp. II	30·1	·42	·623			
				·0379	1·09	2·39
Exp. III	40·3	1·03*	1·01			
						2·84 = mean

* Not including 1st value of K (see Table II).

In Table IV are given the results of a set of experiments made with normal goat-serum and *B. typhosus*. The general character of the action is rather different from that observed with *B. coli commune* and rabbit-serum, the bactericidal action in this case being altogether more powerful. The logarithmic nature of the action is, however, again confirmed, and the effect of temperature is seen to be of the same order, the mean velocity constant being increased 1·93-fold for a rise in temperature of 10° C. between the temperatures of 20·2° and 40·45° C.

The value of this temperature coefficient is lower than that found for most cases of germicidal action by other agents. With phenol, the velocity of disinfection was found, for anthrax spores, to be increased

TABLE IV.

Action of normal goat-serum upon B. typhosus: *effect of temperature.*

Exp. 28. III. 12	Temperature C.	Time, hours	Number of surviving bacteria in 1 standard drop serum = N	$\log_{10} N$	$K = \frac{1}{t_n - t_0} \log \frac{N_0}{N_n}$	$\log_{10} (K \times 10)$	$\log_{10}$ (temperature coefficient per 1° C.)	Temperature coefficient per 1° C.	Temperature coefficient per 10° C.
I	20·2	0·05	526	2·721	($N_0 = 526$; $t_0 = 0·05$)				
		1·3	226	2·354	·29				
		2·08	97	1·987	·36				
		2·7	51	1·708	·38				
		3·8	31	1·491	·33				
		5·0	9	0·954	·36				
		5·8	10	1·000	·39				
		7·8	5	0·699	——				
		9·2	2	0·301	·34 = mean	·531			
							·0291	1·069	1·95
II	30·1	0·05	577	2·761	($N_0 = 577$; $t_0 = 0·05$)				
		1·3	108	2·033	·58				
		2·05	30	1·477	·64				
		2·6	7	0·845	·75				
		3·7	0	—	——				
					·66 = mean	·819			
							·0282	1·067	1·91
III	40·45	0·05	339	2·530	($N_0 = 339$; $t_0 = 0·05$)				
		0·65	40	1·602	1·55				
		1·3	17	1·230	1·04				
					1·29 = mean	1·111		1·068 = mean	1·93 = mean

5·5-fold per 10° C. rise in temperature and as much as 15-fold for *B. paratyphosus*, under certain conditions. Disinfection of the latter organism by higher tar-acids gave a figure of 7–8 per rise of 10° C. (Chick, 1908). In the case of disinfection by hot water the effect of temperature is even more enhanced, the coefficient reaching the high value of 136 per 10° C. rise in temperature for *B. typhosus* (Chick, 1910). Disinfection by means of drying, on the other hand, has a temperature coefficient of only about 2 to 3 per 10° C. (Paul Birstein and Reuss, 1910), and a similar value has been obtained for the germicidal action of metallic salts upon both spores and vegetative organisms (Madsen and Nyman, 1907; Chick, 1908). In the latter case, where there is some evidence of direct chemical union between the salt and the bacterial protein, it is significant that the effect of temperature should be of the same order as that corresponding to most chemical reactions.

The high temperature coefficient of disinfection by hot water recalls a similar effect of temperature upon other reactions where bodies of a protein nature are involved. Such are the destruction by hot water of certain antigens (Famulener and Madsen, 1908) and agglutinins (Madsen and Streng, 1909) and the "heat coagulation" of proteins (Chick and Martin, 1910). In these cases the suggestion has been made that rise of temperature might be accompanied by changes in the nature of the colloidal solution resulting in a greatly increased surface

TABLE V.

Action of normal rabbit-serum (II) *upon* B. coli commune *at* 30° C.*:* *effect of the magnitude of the sowing.*

Exp. I. 8 standard drops (·16 c.c.) of a diluted (1 in 250) paper-filtered culture, added to 2 c.c. serum.

Exp. II. 1 standard drop (·02 c.c.) of a diluted (1 in 250) paper-filtered culture, added to 5 c.c. serum.

Exp. 13. III. 12	Time, hours $= t$	Amount of sample taken, no. of drops	Numbers counted on plates		Mean no. of bacteria surviving in 1 standard drop of serum $= N$	$\text{Log}_{10} N$	$K = \frac{1}{t_n - t_0} \log \frac{N_0}{N_n}$
I	0·08	1	1890		1890		($N_0 = 2230$; $t_0 = 4·8$)
	0·66	1	1600		1600		
	2·0	1	1960		1960		
	2·85	1	3310		3310		
	4·8	1	2250	2210	2230	3·348	
	5·8	1	341		341	2·533	·81
	6·5	1	147		147	2·167	·69
	7·0	1	94		94	1·973	·62
	8·05	1	49		49	1·690	·51
	24·0	5	0		0		
							·66 = mean
II	0·08	8	719		90·9		($N_0 = 104·5$; $t_0 = 4·7$)
	0·66	5	430		86		
	2·0	5	682		136		
	2·85	5	1142		228		
	4·7	3 5	281 555		104·5	2·019	
	5·8	5	56		11·2	1·049	·88
	6·5	3	17		5·4	0·732	·71
	7·0	5	2		0·4		
	8·1	1 3	0 1		0·2		·79 = mean
	24·0	10	0		0		

for action. In disinfection by hot water some such hypothesis might also explain the enhanced effect of temperature. The comparatively small influence of temperature in the present instance indicates that the mechanism involved in the germicidal action of serum must be of a different and probably simpler character. At the same time it is necessary to point out that a fallacy may be involved when attempts are made to elucidate the nature of a process by such indirect means as the study of the temperature effect. The value of the temperature coefficient need not necessarily refer to the essential part of the process but only to some other factor limiting the velocity of the action without necessarily being closely involved in it.

Effect of magnitude of the sowing.

The results of Tables I–IV received further confirmation in the case of experiments specially undertaken to investigate other points.

For example, Exps. I and II in Table V were made in order to ascertain what influence the initial number of bacteria added might have upon the bactericidal action of the serum. The data are somewhat scanty, but show approximately the logarithmic nature of the process. In the case of the heavier sowing, a concentration of about 1900 bacteria per drop, disinfection appears to be distinctly retarded, but the effect is not very marked when the mean value of the velocity constant, 0·66, is compared with that for an initial concentration of bacteria of only 90 per drop, viz. 0·79.

Influence of previously heating the serum.

A comparison of Experiments A and B in Table VI show the effect upon bactericidal action of previously heating the serum to 55–57° C. for about 20 minutes. As would be expected, the bacteria experienced no germicidal action in Exp. B with heated serum and, after a latent period which lasted about one hour, underwent steady multiplication.

The rate of multiplication was measured by calculating the mean generation time. If $T =$ the mean generation time, and N_n and $N_0 =$ the number of bacteria present in unit volume after times t_n and t_0 respectively, then, assuming cell-division to be steadily and universally maintained, the number of successive cell-divisions taking place in the interval of time $t_n - t_0$ will be equal to

$$\frac{t_n - t_0}{T}$$

and $N_n = N_0 \times 2^{\left(\frac{t_n - t_0}{T}\right)}$

or $\log N_n - \log N_0 = \frac{t_n - t_0}{T} \log 2$

and $T = \frac{\log 2}{\frac{\log N_n - \log N_0}{t_n - t_0}}$,

the denominator of which fraction has been represented by A and called the "growth constant."

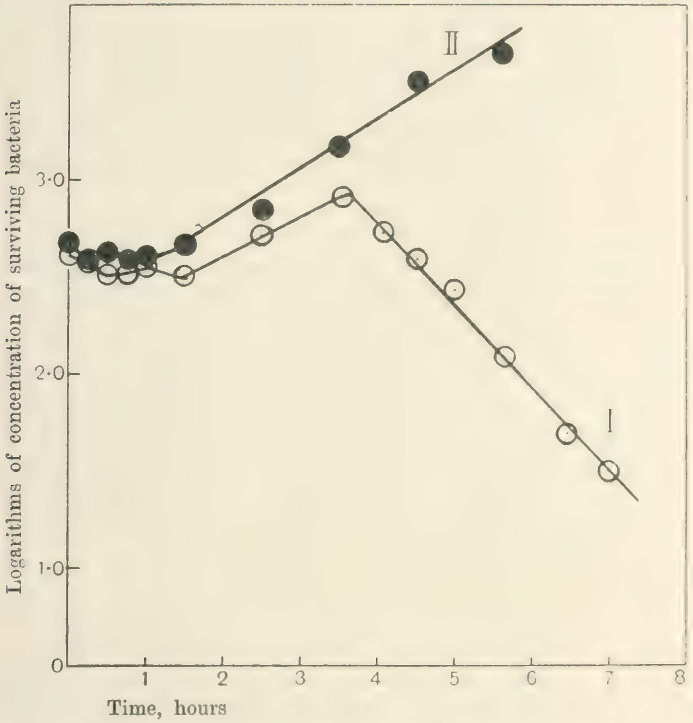

Fig. 3. Action of normal rabbit-serum upon *B. coli commune* (see Table VI).

I. Serum not heated.

II. Serum previously heated to 55–57° C. for 20 minutes.

The values of T were calculated up to 5·6 hours in Exp. B and show a fair constancy considering the great difficulty experienced in counting the very crowded plates with any accuracy. The mean generation was 1·4 hours or 84 minutes.

In Exp. A, made with unheated serum to form an exact control for B, the initial latent-period also lasted about one hour. In the period of growth which followed it is interesting to notice that the calculated value of the generation-time is not very dissimilar to that obtained in Exp. B. In fact when allowance is made for the difficulty of setting boundaries to the various stages in Exp. A, each of which is of short duration, the agreement in the two experiments is surprising. For the first three and a half hours the course of events runs almost parallel in the two cases.

This is well shown in Fig. 3, where logarithms of concentration of living bacteria are plotted against time. The same curve shows the logarithmic nature of the subsequent disinfection in Exp. A to be well maintained for a period of about four hours after which there appears to be some discrepancy. The values of the velocity constant, given in the last column of Table VI, also show a falling off towards the end of the experiment. It should be noted, however, that the concentration of survivors have been reduced so low that the enumerations are not very trustworthy.

The results of a second experiment are set out in Table VII. It was less complete than that of Table VI, and the numbers obtained on the plates were too great for accurate counting. The calculations must be regarded as approximate only, but serve as useful confirmation of the previous results. In this case in Exp. A, between the sixth and tenth hour, after the majority of the bacteria had been killed, the residue began to multiply. The rate of growth was similar to that obtaining in the short period of growth (second to fourth hour) previous to the disinfection, and also similar to that steadily maintained throughout in Exp. B with the heated serum.

Influence of temperature upon growth in serum.

The temperature coefficient of bacterial growth in serum was investigated in material previously heated to 55–57° C. for about 20 minutes. Three similar experiments at different temperatures are given in Table VIII and graphically shown in Fig. 4. They all show a preliminary latent period followed by regular multiplication which was maintained as long as the observations could be made. In Table IX are given the results of two experiments made at the same time at 30·1° C. with nutrient broth and normal saline solution respectively for comparison with Exp. II in Table VIII. By comparing the calculated values of the generation-time 0·54 hours (or 32 minutes) in broth and 0·69 hours (or 41 minutes) in serum, it will be seen that multiplication took place rather more slowly in the latter medium. The values of the

TABLE VI.

Action of normal rabbit-serum (I) *upon* B. coli commune *at* 30·3° *C.*

A. Serum unheated.

B. Serum previously heated for 20 minutes at 55–57° C.

Two standard drops (0·04 c.c.) of a 24 hours' culture of *B. coli commune* in broth, diluted 120-fold in distilled water, added to 3 c.c. serum.

Exp. 28. II. 12	Time, hours $=t$	Amount of sample taken, no. of drops	Numbers counted on plates		Mean no. of surviving bacteria in 1 drop serum $=N$	$\text{Log}_{10} N$	Growth constant $A = \frac{1}{t_n - t_0}\log\frac{N_n}{N_0}$	Generation time, hours $=\frac{\log 2}{A}$	Disinfection constant $K = \frac{1}{t_n - t_0}\log\frac{N_0}{N_n}$
A	0·03	1	409	402	405	2·607			
	0·25	1	391	359	375	2·574			
	0·5	1	332	313	322	2·508			
	0·75	1	322	330	326	2·513			
	1·0	1	350	366	358	2·554	($N_0 = 327$;		
	1·5	1	332	322	327	2·514	$t_0 = 1·5$)		
	2·5	1	502	546	524	2·719	·205	1·47	($N_0 = 813$;
	3·55	1	816	810	813	2·910	·193	1·56	$t_0 = 3·55$)
	4·07	1	532	538	535	2·728			·35
	4·5	1	396	392	394	2·595		1·51 = mean	·33
	5·0	1	263	276	269·5	2·431			·33
	5·66	1	117	123	120	2·079			·39
	6·45	1	57	42	49·5	1·695			·42
	7·0	1	29	33	31·5	1·498			·41
	8·25	1	30	20	25	1·398			·32
	9·1	1	16	18	17	1·230			·30
	10·25	1	17		11·8	1·072			·27
		5	54						
	11·4	1	6		5·3	0·724			·28
		5	26						
	13·6	1	2	1	1·0	·00			·29
		5	4						
	18·0	1	0	(?) 1	0				·34 = mean
		5	0						
	24·0	1	0		0				
		4	0						
	31·0	1	0		0				
		4	0						
B	0·03	1	468	476	472	2·674			
	0·25	1	377	389	383	2·583			
	0·5	1	430	416	423	2·626			
	0·75	1	386	387	386	2·587			
	1·0	1	412	377	395	2·597	($N_0 = 444$;		
	1·5	1	453	435	444	2·647	$t_0 = 1·5$)		
	2·5	1	690	718	704	2·848	·201	1·50	
	3·5	1	1572	1368	1470	3·167	·260	1·16	
	4·5	1	3152	3240	3200	3·505	·286	1·05	
	5·6	1	4052	4928	4490	3·652	·245	1·23	
								1·26 = mean	

TABLE VII.

Action of normal rabbit-serum (I) *upon* B. coli commune *at* 30·3° *C.*

A. Serum unheated.

B. Serum previously heated for about 25 minutes at 55–56° C.

Two standard drops (0·04 c.c.) of a 24 hours' culture of *B. coli commune* in broth, diluted 60-fold in distilled water, added to 3 c.c. and 2·7 c.c. serum in Experiments A and B respectively.

Exp. 20. II. 12	Time, hours = t	Amount of sample taken, no. of drops	Numbers counted on plates		Mean no. of surviving bacteria in 1 drop serum = N	$\log_{10} N$	Growth constant $A = \frac{1}{t_n - t_0} \log \frac{N_n}{N_0}$	Generation time, hours $= \frac{\log 2}{A}$	Disinfection constant $K = \frac{1}{t_n - t_0} \log \frac{N_0}{N_n}$
A	0·05	1	1336	1412	1370		(N_0 = 1460;		
	1·0	1	1388	1532	1460	3·164	t_0 = 1·0)		
	2·0	1	2120	2412	2270	3·356	·192	1·57	
	3·0	1	6888	6064	6480	3·812	·324	0·93	(N_0 = 7810;
	4·33	1	7808		7810	3·893	·219	1·37	t_0 = 4·33)
	6·4	1	1396	1408	1400	3·146	(N_0 = 1400; t_0 = 10·6)		·36
	10·6	1	16770	24350	20560	4·313	·278	1·08	
								1·21 = mean	
	12·1	1	x						
B	0·05	1	1460	1512	1490				
	0·86	1	1660	1680	1670		(N_0 = 1970;		
	1·9	1	1840	2092	1970	3·294	t_0 = 1·9)		
	3·0	1	3640	4152	3900	3·591	·270	1·11	
	4·25	1	8064	7712	7890	3·897	·257	1·17	
	6·33	1	18944	19584	19260	4·285	·224	1·34	
	10·6	1	x					1·21 = mean	

TABLE VIII.

Effect of temperature upon growth of B. coli commune *in normal rabbit-serum* (I) *previously heated to* 55–57° *C. for* 20 *minutes.*

Exp. 6. III. 12	Temperature, C.	Time, hours = t	Amount of sample taken, no. of drops	Numbers counted on plates		Mean no. of living bacteria in 1 standard drop serum N	$\log_{10} N$	Growth constant $A = \frac{1}{t_n - t_0} \log \frac{N_n}{N_0}$	Generation time, hours $= \frac{\log 2}{A}$
I	19·8	0·08	5	139	111	25	1·398	(N_0 = 48·3;	
		2·3	5	120	105	22·5	1·352	t_0 = 6·4)	
		4·3	4	119	110	28·6	1·456		
		6·4	2 4	90 200		48·3	1·684		
		8·4	2	247	269	129	2·111	·213	1·41
		10·0	1 3	550 848		349	2·543	·239	1·26
		11·7	1	619	647	633	2·801	·211	1·43
									1·37 = mean
II	30·1	0·08	5	117	103	22	1·342	(N_0 = 56·4;	
		2·3	5	293	271	56·4	1·751	t_0 = 2·3)	
		4·3	4	2136	1748	485	2·686	·467	·645
		6·4	2	*9376	8160	4380	3·641	·461	·653
		8·4	2	x					·649 = mean
III	34·9	0·08	5	90	112	20·2	1·305		
		1·0	5	119	113	23·2	1·365		
		2·3	2 3	174 324		99·6	1·998	(N_0 = 99·6; t_0 = 2·3)	
		4·3	2	3792	3776	1890	3·276	·639	·471
		6·3	1	*38620	34620	36620	4·564	·641	·470
									·470 = mean

* Numbers must be regarded as approximate.

generation-time are calculated on the assumption that multiplication is universal among the bacteria and maintained without interruption. But it may well be that in serum the longer generation-time is to be

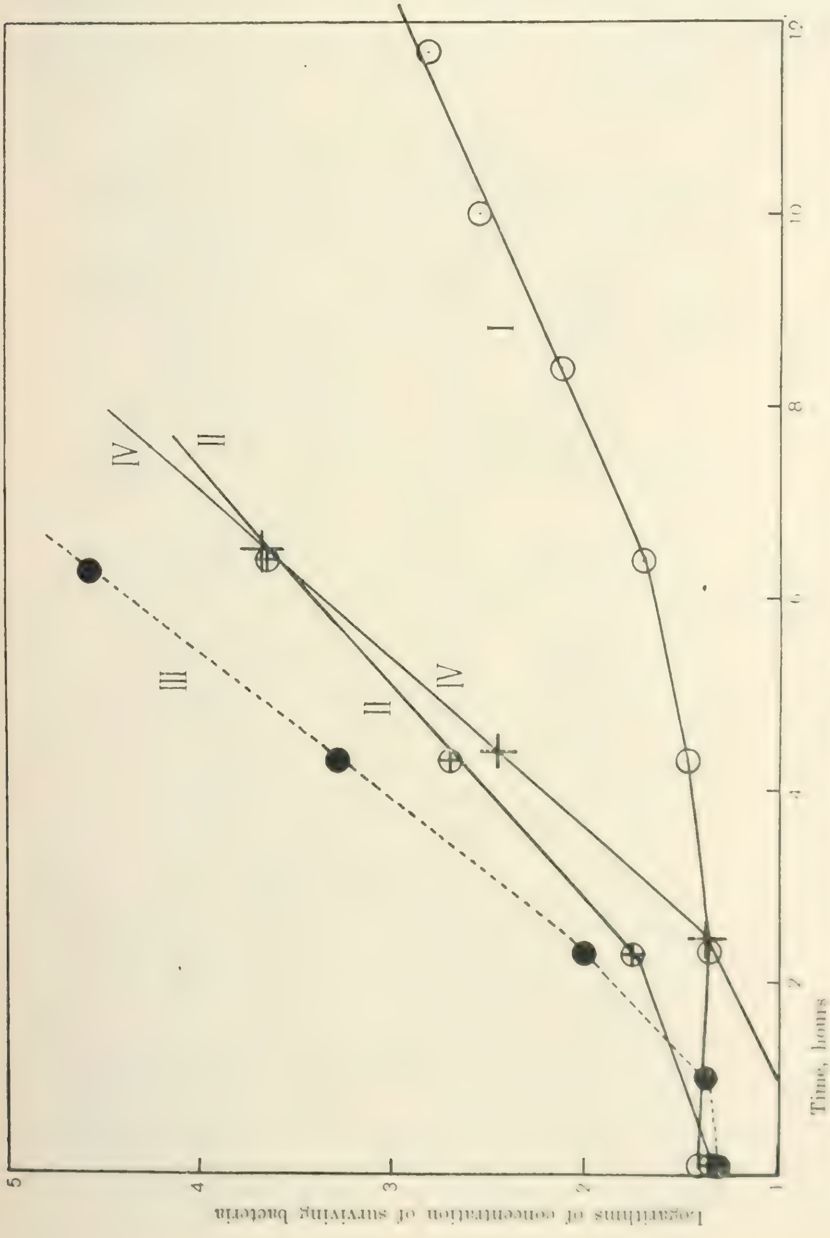

Fig. 4. Influence of temperature upon the growth of *B. coli commune* in normal rabbit-serum, heated to 55–57° C. for 20 minutes (see Table VIII).

I. at 19·8° C. II. at 30·1° C. III. at 34·9° C. IV. in broth at 30·1° C.

explained by the fact that some individuals do not multiply at all, or that some are dying while others are growing. The point could be settled by a determination of the generation-time by direct observations under the microscope, using a dark ground and side illumination, and it is hoped subsequently to publish the results of a comparable set of experiments to the present in which the microscopic method is substituted for that of plate cultures.

TABLE IX.

Growth of B. coli commune *in (a) nutrient broth, and (b) "normal" saline solution at* 30·1° *C., for comparison with Exp. II, Table VIII.*

Exp. 6. III. 12	Time, hours t	Amount of sample taken, no. of drops	Numbers counted on plates		Mean no. of living bacteria in 1 standard drop $=N$	$\text{Log}_{10} N$	Growth constant $A = \frac{1}{t_n - t_0} \log \frac{N_n}{N_0}$	Generation time, hours $= \frac{\log 2}{A}$
(*a*)	0·05	5	43	29	7·2		($N_0 = 23·4$;	
	2·45	5	123	111	23·4	1·369	$t_0 = 2·45$)	
	4·4	2	462	648	277	2·443	·551	·546
	6·5	2	8430	10210	4660	3·668	·568	·530
	23·1	·002 (by dilution)	*12580	13250	6457000	6·810		·538 = mean
(*b*)	0·05	5	28	52	8		($N_0 = 28·4$;	
	1·3	5	57	56	11·3		$t_0 = 6·5$)	
	2·45	5 10	60 152		14·1			
	4·43	5	64	66	13			
	6·5	5	154	130	28·4	1·453		
	8·5	5	260	256	51·6	1·713	·130	
	10·03	5 10	401 794		79·7	1·901	·127	
	11·8	10 17	976 1940		108·1	2·034	·110	
	22·8	10	6180	6020	610	2·785		

* Number must be regarded as approximate.

The figure for generation-time in broth at 30·1° C., viz. 32 minutes, is in fair agreement with that obtained for *B. coli commune* by Lane-Claypon (1909) using a similar method to the above, viz. 31·8 minutes at 31° C. The fact that the value is also consistent with that obtained by direct observation of the bacterial cells (Barber, 1908, Table II, p. 389), viz. 30·7 minutes at 30° C., goes to prove that during the early hours of a broth culture multiplication is universal. Whether multiplication in serum is really slower or is only less general can only be settled by further experiment.

The effect of temperature was measured by comparison of "growth constants" at the various temperatures of experiment. These are set out in Table X and the influence of temperature is seen to be fairly consistent and in accordance with a logarithmic law. It is not possible to decide whether the velocity of growth is influenced by rise of temperature in accordance with the law of Arrhenius or in a simple logarithmic manner, for over a small range of temperature the two expressions become almost identical. The same is true of the influence of temperature upon the disinfectant action of serum.

TABLE X.

Temperature coefficient of growth of B. coli commune *in normal rabbit-serum* (I) *heated* (*see Table VIII*).

Exp. 6. III. 12	Temperature °C.	Mean value of the growth constant A	Log_{10} ($A \times 10$)	Log_{10} (temperature coefficient per 1° C.)	Temperature coefficient per 1° C.	Temperature coefficient per 10° C.
I	19·8	·221	·344			
				·0313	1·074	2·06
II	30·1	·463	·666			
				·0292	1·070	1·96
III	34·9	·640	·806			
					1·072 = mean	2·01 = mean

The rate of growth in serum is influenced by temperature to a degree comparable with that shown in the case of the death-rate (see above), being increased 1·07-fold and 2·01-fold for a rise of temperature of 1° C. and 10° C. respectively, between the temperatures of 20° C. and 35° C. That the effect remains consistent at least as high as 40° C. may be gleaned from the somewhat scanty data of the growth phase in Exps. I, II and III, Table II.

These values are in close accord with those obtained for growth of *B. coli* in broth by Lane-Claypon and Barber respectively. The former worker found a temperature coefficient of 2·13 per 10° C. between the temperatures of 20° C. and 34° C. (calculated from data given in Table I, *see* Lane-Claypon, 1909, p. 246) and the latter obtained 2·36 per 10° C. between 20° C. and 35° C. (from curve, *see* Barber, 1908, p. 696).

Summary.

1. The action *in vitro* of normal rabbit-serum upon *B. coli commune* consists usually of three or more phases, the duration of which is inversely related to temperature.

2. The latent period which occurs on first mixing bacteria and serum is followed by a period of bacterial growth, which in its turn

yields to a period of bactericidal action. This may remain final or may be succeeded by a second period of growth.

3. The germicidal action, both in case of normal rabbit-serum upon *B. coli commune* and of normal goat-serum upon *B. typhosus*, takes place in accordance with a logarithmic law so that death-rate remains proportional to the concentration of surviving bacteria.

The bactericidal action of serum thus falls into line with all other cases of disinfection, hitherto investigated.

4. The temperature coefficient of the bactericidal action of serum is low in comparison with that found for other germicides, being 2·84 per 10° C. rise in temperature for normal rabbit-serum and *B. coli commune* and 1·93 in the case of normal goat-serum and *B. typhosus*.

5. Growth of *B. coli commune* in rabbit-serum takes place logarithmically so that the concentration of living bacteria after equal intervals of time form terms of a geometrical series.

The calculated value of the generation-time is slightly greater than that obtaining in broth at the same temperature.

6. The influence of temperature upon rate of bacterial growth in serum is comparable with the effect upon growth in other media. The generation-time is decreased 2·01-fold for a rise in temperature of 10° C.

7. The separate processes of bacterial growth and death in serum being similarly influenced by temperature, there is no support for the view that the significance of fever might be concerned with a tendency to encourage either process relative to the other.

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IDIOCY AND CONGENITAL SYPHILIS.*

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The question of the importance of syphilis in the parents as a cause of mental deficiency in the offspring has led to the expression of very divergent opinions. The majority of authorities appear to have held that syphilis was a relatively unimportant factor. Others from a study of cases which have come under their notice have inclined to the view that syphilis was a very potent cause in the production of idiocy.

E. Mendel (1868) quoted an interesting case described by Carl Friedrich Haase in 1828. A young woman who had been infected by her husband had three miscarriages. The fourth pregnancy resulted in the birth of a living child which died with marked hydrocephalus at the age of seven months. After quoting the opinions of several writers Mendel remarks that "clinical observations in this connection are limited to a few cases, the number of which stands in marked contrast to the frequency with which pathological changes are found in the brains of children who succumb to hereditary syphilis. In any case the majority of hereditary syphilitics die long before there can be any question of mental deficiency. In those cases, however, in which hereditary syphilis is latent or cured, but which at a later period develop symptoms of mental disturbance, it is generally impossible to refer such disturbance to hereditary syphilis, for the objective symptoms of this disease are then variable, indefinite and in no sense specific." Mendel, as may be seen from this extract, saw very clearly the difficulties which beset the proof of the view which he adopted.

It was not, however, generally considered that syphilis was a very important factor in the causation of idiocy. Critchett in 1860 stated that "there does not appear to be any reason for supposing that actual idiocy is at all a common consequence of inherited syphilis. About three years ago we looked through the patients at the Redhill Asylum without finding a single one whose teeth were characteristic, and Dr. Down informs us that he has recently made another inspection with a like result."

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* A paper read at a Special Meeting on Syphilis at the Royal Society of Medicine on July the 8th, 1912.

Sir Jonathan Hutchinson (1863) found an idiotic condition in only three out of 170 cases of congenital syphilis. The same observer (1879) examined the teeth of the idiots at the Earlswood Asylum but did not find the specific characteristics in any considerable number of cases.

Kerlin (1880) made a careful inquiry into the histories of the parents and grandparents of 100 cases of idiocy. In only 2 cases was a history of syphilis elicited, while 4 only of the 100 patients showed any evidence of a syphilitic infection.

Judson Bury (1883) described 6 cases of mental deficiency in children who were the subjects of congenital syphilis. He came to the conclusion " that hereditary syphilis is a more frequent factor in the production of mental deficiency than has hitherto been recognised."

Shuttleworth (1888) examined the records of 1000 cases at the Royal Albert Asylum at Lancaster. In 10 cases only was there reason for suspecting inherited syphilis, and in 4 cases only could the evidence be called satisfactory. Shuttleworth, however, expresses the following opinion : " In spite of these figures I am inclined to believe that inherited syphilis plays a larger part in the production of mental enfeeblement in childhood than institution statistics would lead us to suppose."

A few cases were recorded by Fletcher Beach (1888), who, however, concluded that " syphilis is not a common exciting cause of imbecility."

Down (Lettsomian Lectures, 1887), in discussing the causes of idiocy, states that in not more than 2 per cent. of idiots were there signs of inherited syphilis.

Piper (1893) examined 316 idiots and found evidence of syphilis in 5 per cent.

Hahn (1898) examined 540 idiots at the Alsterdorfer Asylum ; of these, 40 (7·4 per cent.) were found to be syphilitics. Of 100 deaf-mutes at the Hamburg Asylum 4, and of 60 blind children 10, showed evidence of congenital syphilis.

Wachsmuth (1905) was unable to find even one congenital syphilitic among 185 idiots whom he examined.

Ziehen (1908) records the result of a careful statistical inquiry as to the frequency of congenital syphilis in cases showing a slight degree of mental deficiency. Ziehen found that syphilis was probably present in 17 per cent. and certainly present in 10 per cent. of the cases examined.

A very different result was recorded by Shuttleworth and Fletcher Beach, who could only trace inherited syphilis in 1·17 per cent. of their cases.

From this review of the subject it will be seen that a very wide divergence of opinion exists. It appears, however, to be quite certain that only a small proportion of idiots show the classical signs and symptoms of congenital syphilis, and if we are restricted to the usual methods of examination we must come to the conclusion that congenital syphilis has little connection with idiocy. On the other hand, the view that congenital syphilis is an important cause of idiocy has been put forward by several writers. This opinion has been based for the most part on cases of idiocy, in which, while the usual signs of syphilis have been wanting in the patient, it has been possible to establish a definite history of syphilis in the parent. The number of such recorded cases is, however, small.

The opportunity of collecting further evidence was afforded by the introduction of the Wassermann test. In the five years which have elapsed since the discovery of the Wassermann test, it has been established beyond all reasonable doubt that a positive reaction may be regarded as conclusive evidence of a syphilitic infection. It is stated that positive reactions have been obtained with the sera of patients suffering from framboesia, trypanosomiasis, leprosy, and in certain cases of malaria. These diseases are, however, of rare occurrence in this country, and we may safely conclude that a patient whose serum gives a positive Wassermann reaction has certainly been infected with syphilis. The Wassermann reaction is, moreover, a remarkably constant sign of syphilis, for it is well known that syphilitic patients during a latent stage, at a time when no sign or symptom of the disease is apparent, may give a definite reaction to the serum test. By the aid of the serum reaction we are able to detect syphilis in an individual who not only may show no sign of syphilis but may appear to be in perfect health. Still more remarkable are those cases in which a positive serum reaction is from first to last the only evidence of infection. It has been established that mothers of syphilitic children who appear to be in perfect health and who have never shown any sign of syphilis give in the large majority of cases a positive reaction to the serum test. The work of the last five years has established two very important facts:

(1) A positive Wassermann reaction (if certain diseases, rare in this country, can be excluded) may be accepted as specific evidence of syphilis.

(2) A positive reaction may be obtained with the serum of an individual who shows no other evidence of syphilis.

If we are prepared to accept the truth of these two statements, it is obvious that we have in the serum test an invaluable aid in determining the ætiology of a variety of morbid conditions.

The examination of the blood-serum of idiots by the Wassermann reaction has been the subject of several papers.

Raviart, Breton, Petit, Gayet and Cannac (1908) examined 246 cases, of which 76 (a little more than 30 per cent.) were found to give a positive reaction.

Kellner, Clemenz, Brückner and Rautenberg (1909) examined 216 cases, of which 13 gave a positive reaction by Stern's method, while 9 only gave a positive reaction by the original Wassermann method. To the 13 cases must be added 3 cases which were deficient in complement and were found to be positive by the original Wassermann method (7·4 per cent.). It should be noted that of these 216 cases half were over thirty and 40 cases only were under fourteen years of age. Of the patients under fourteen years of age 20 per cent. gave a positive result, of those over fourteen only 5 per cent.

Lippmann (1909) examined 78 cases at the Uchtspring Asylum, and obtained a positive reaction in 7 cases (9 per cent). An examination of the cases at the Dalldorf Asylum gave a positive result in 13·2 per cent. Lippmann also examined 77 cases by clinical methods, and decided that 40·2 per cent. showed signs of congenital syphilis.

In 1909 I had the opportunity of examining 330 of the inmates of the Wilhelmstift, an asylum for idiots at Potsdam. A positive result was obtained in 51 cases (15·4 per cent.). Among the 51 cases which gave a positive reaction 7 were found which had definite signs of syphilis, and 3 or 4 in which syphilis might have been suspected but not with certainty diagnosed. There were 2 cases with definite signs which gave a negative reaction. That is to say, among the 330 patients were 9, or including doubtful cases, 13, which from physical signs and symptoms would have justified the diagnosis of syphilis. The results in detail were as follows:

	Cases examined.	Positive.
Simple idiocy of all grades	287	44
Congenital spastic diplegia (Little's disease)	15	1
Marked hydrocephalus	14	4

	Cases examined.	Positive.
Epilepsy	1	1
Microcephalic cases	4	0
Mongols	1	0
Deaf and dumb	7	1
Progressive muscular dystrophy, with mental symptoms	1	0

All the cases which gave a positive serum reaction were subsequently very carefully examined with the object (1) of discovering any sign of syphilis which had been previously overlooked, and (2) of detecting any symptom or group of symptoms common to all the positive cases. Of the positive cases, one was subject to epileptiform convulsions and showed slight choreic movements, one had strabismus and nystagmus, one had a right-sided hemiplegia, one had spastic diplegia and conformed to the type of Little's disease, one was a deaf-mute, and two were aphasic. Among the remaining cases I was unable to detect any evidence of a local lesion.

An examination was made of the cerebro-spinal fluid from 12 cases which had given a positive serum reaction. In only one case was a positive reaction obtained. I also obtained for examination specimens of serum from the parents of ten of the positive cases. The results were as follows:

Patient.	Age of patient.	Result of examination of	
		Father's serum	Mother's serum.
B—	9	Positive	Positive
Har—	15	Not examined	Positive
Kr—	11	Positive	Positive
M—	11	Positive	Not examined
V—	13	Not examined	Positive
O—	11	Negative	Not examined
Sc—	16	Positive	Not examined
R—	12	Negative	Not examined
N—	9	Negative	Negative
He—	14	Not examined	Positive

Thus among 13 parents of children giving a positive reaction, 9 were found to give a positive reaction. Six mothers were examined and 5 gave a positive reaction. Seven fathers were examined, and in 4 cases a positive reaction was obtained.

It will be noticed that in the above table in the case of the patient Har— a positive reaction was obtained at an interval of fifteen, and the case of Sc— at an interval of sixteen, years after the birth of a syphilitic child. The period during which a positive reaction may be obtained is known to be extremely variable in the case of the acquired form of the disease. In the congenital form it might be expected that the percentage of positive results would bear a close relation to the age of the patients examined. A grouping of the 330 cases according to age gives the following result:

	Examined.	Positive.	Percentage of positive results.
(1) Patients aged 10 years and under (of these two only were less than 5 years old)	94	20	21·27
(2) Patients from 11 up to 15 years of age inclusive	142	24	16·9
(3) Patients from 16 to 20 years of age	66	4	6·06

Of patients aged from 21 to 30, 24 were examined with 3 positive results. The remaining 8 patients ranged in age from 31 to 44 and all 8 gave a negative reaction.

The above table appears to show that the percentage of positive results diminishes rapidly after the sixteenth year, and that a larger percentage of positive results might be expected from the examination of a series of very young cases. In any case the average age of the patients investigated must be regarded as an important factor in any estimation of the prevalence of congenital syphilis, and it seems to me possible that the very contradictory results already published may be reconciled by taking the age-factor into consideration. Of the 51 cases in which a positive serum reaction was obtained 7 only showed conclusive evidence of congenital syphilis from a clinical standpoint. In the remaining 44 cases a diagnosis of syphilis rested on the evidence of the serum test.

I had hoped that by a careful examination of those cases which had given a positive result to the serum test it might be possible to detect some symptom or group of symptoms which was common to all. This I failed to do. Very few of the positive cases showed any evidence of a gross lesion in the central nervous system; and this, I think, is quite in accordance with what one might expect, for the gross changes in the brain which are known to be due to congenital syphilis are not compatible, as a rule, with a continuance of life. If a causal relation exists between congenital syphilis and idiocy, the condition which arises may perhaps be classed as parasyphilitic. The absence of the ordinary signs of congenital syphilis in idiocy is closely paralleled in the already authenticated parasyphilitic diseases. It is, of course, well known that tabes and general paralysis commonly occur in patients where the early symptoms of syphilis have been mild or even unnoticed. Among the cases of the juvenile form of general paralysis collected by Mott (1909) quite half were found to show no sign of congenital syphilis, but nevertheless to have been born of syphilitic parents and to have brothers and sisters who exhibited the ordinary signs of the disease.

It seems to me reasonable to think that many cases of idiocy should be classed with that form of syphilis which manifests itself alone by a selective toxic action on the elements of the central nervous system. I do not wish to attach an exaggerated importance to the results of the examination of the serum in one series of cases, but when it can be shown that a considerable percentage of idiots afford evidence of a syphilitic infection, and since it is well known that the virus of syphilis is capable of exercising a selective action on the central nervous system in cases in which there is no other evidence of the disease, I think it is not unreasonable to infer a causal relation between the two conditions.

Further evidence has been furnished by Atwood (1911), who examined 204 cases in America. A positive result was obtained by Noguchi's method in 30 cases (14·7 per cent.). The results of an examination of three cases which gave positive reactions were published by Bellingham Smith and Woodford (1911), and Chislett (1911) examined 14 idiots, 8 of whom gave a positive and 6 a negative reaction. Chislett also records the results of an examination of an entire family. The father had general paralysis of the insane and the Wassermann reaction was positive. The mother had no knowledge of the occurrence of any primary or secondary symptoms. She had had a tertiary ulcer on the left leg eight years after marriage. The eldest son, aged 16 years, was said to have been "very nervous and stupid at school." He showed no sign of syphilis, but gave a positive reaction. A girl, aged 12 years, was deaf in one ear, but otherwise normal. Her serum gave a positive reaction. A girl, aged 10 years, gave a negative reaction and appeared to be quite normal. A boy, aged 8 years, had rhinitis and conjunctivitis and gave a positive reaction. The two youngest children, aged 6 and 4 years respectively, were healthy and gave a negative reaction.

Knöpfelmacher and Schwalbe (1912) examined 29 cases of hydrocephalus, 8 of which gave a positive reaction.

The results obtained in Denmark by Thomsen, Boas, Hjort and Leschly (1911) were very different. These workers examined 2044 feeble-minded persons, of which only 31 (1·5 per cent.) gave a positive reaction. On the other hand, E. Krober (1911) examined 262 idiots at the Hephata Asylum at Gladbach and obtained a positive reaction in 21·4 per cent.

Although the results obtained by different workers in different countries are not in entire agreement, sufficient evidence has accumulated to make it appear extremely probable that congenital syphilis is an extremely important cause of mental deficiency. It must be remembered that the majority of idiots who give a positive Wassermann reaction show no other sign of syphilis. They are, in fact, cases of latent syphilis, and we are aware that during the latent stages of the acquired disease only some 40 to 50 per cent. of the patients give a positive reaction. I am therefore inclined to think that the actual percentage of positive results obtained by examining a series of idiots by the serum test comes very far short of the number which are actually infected. If this assumption is correct, we shall have to recognise syphilis as the causative factor in a very considerable percentage of cases of idiocy. The problem appears to me to be worth further effort, and an examination of another large series of cases could hardly fail to afford interest. Particularly valuable information would doubtless be forthcoming if it were found possible to make an examination of all the members of a family in which a case of idiocy had occurred. In any case the results which I obtained in Berlin show that it is desirable to examine the blood-serum at the earliest possible age. The low percentage of positive cases obtained by some workers must in all probability be attributed to the fact that a large number of adult patients have been included in their results. I am inclined to think that an examination of a series of very young patients will give a very high percentage of positive results. Additional and valuable evidence will no doubt be obtained if it is found possible to include an examination of the blood of the parents.

We may classify the cases in which congenital syphilis is the cause of disease of the nervous system into three groups :

(1) The first group includes infants who are born with marked evidence of syphilis. In these cases the brain, together with other organs of the body, is the seat of marked change. These children as a rule die within a short time of their birth.

(2) This group includes children who appear to be healthy at birth but which develop mental defects at the time of the second dentition or at puberty. To this group belong the cases of juvenile general paralysis.

(3) In this group may be placed those cases of mental deficiency,

imbecility and idiocy in many of which the Wassermann reaction constitutes the sole evidence of a syphilitic origin.

It is well known that in many cases the syphilitic virus appears to exert a selective toxic action on the central nervous system, and we might expect this toxic action to exert its influence most injuriously during that period when the brain, the most highly specialised organ of the body, is undergoing development.

Syphilis is a wide-spread disease and there is no reason for supposing that its prevalence is on the wane. On the other hand, there is a very general belief that the type of the disease is changing. While the more obvious and gross manifestations of the disease are less often seen, there is reason to think that parasyphilitic disease is increasing. The connection between syphilis and general paralysis of the insane has been established. Is it not reasonable to think that further research may establish a connection between syphilis and other diseases, which at the present time are not suspected of a syphilitic origin? The remote effects of syphilis may be even more numerous than we suppose. Organs other than the brain and spinal cord may be the seat of parasyphilitic disease. The Wassermann reaction affords a method by which this problem can be attacked, but it is obvious that results obtained by the serum test must be subjected to rigorous criticism. The occurrence of a positive Wassermann reaction in any one patient is in itself no proof that the condition which it is desired to investigate has been caused by syphilis. The presence of a syphilitic infection in such a case may be a mere coincidence. It is only after a very large number of cases have been investigated that the coincidence of a certain group of symptoms with a positive Wassermann reaction can justify us in forming an opinion. Such an investigation must be carried out on a large scale, for the examination of a very large number of patients is a necessary step to obtaining the required statistics. A commencement has been made, and the results obtained by the examination of patients with aortic disease may be here quoted.

Donath (1909) examined 27 cases of aortic insufficiency, aortitis and aneurysm of the aorta. A positive serum reaction was obtained in 85 per cent. of the cases.

Bruckner and Galasesco (1910) obtained a positive reaction in 17 of 22 cases of aortic regurgitation.

Longcope (1910) obtained a positive reaction in 18 of 22 cases of aortic regurgitation.

These results are perhaps sufficient to show the possibilities of a systematic use of the serum test. The cases examined have, however, been too few for the results to be of real value, and in the majority of instances only those patients have been examined in whom a suspicion of syphilis was entertained.

An inquiry on broader lines is needed, and such an investigation should include the examination of cases in which syphilis is not suspected. By such means it might prove possible to enlarge our knowledge of the pathological changes produced by syphilis. An investigation on these lines has been undertaken by Churchill (1912), who has examined 102 infants and children, patients at a children's hospital in America. A positive Wassermann reaction was obtained in no less than 39 cases.

As regards the treatment of congenital syphilis it is obviously desirable that it should be commenced at the earliest possible moment. The serum test affords a means of early diagnosis and leads to the treatment of children in whom the disease is latent during the early years of life. Valuable information can without doubt be obtained by examining the blood of the mother during pregnancy.

Knöpfelmacher and Lehndorff (1910), who have devoted particular attention to this subject, have examined 135 mothers of syphilitic children and have obtained a positive result in 65·2 per cent. Of these 135 mothers 31 only showed definite signs of syphilis, while in the remaining 104 a diagnosis was only possible as a result of the serum test.

Stroscher (1910) obtained a positive result in 100 per cent. of the mothers of syphilitic children.

Mulzer and Michaelis (1910) found that 96 per cent. of infants with manifest congenital syphilis gave a positive reaction, while children with latent syphilis react like adults in the early latent period. They also found that 83 per cent. of mothers of syphilitic children gave a positive reaction. In an earlier paper Knöpfelmacher and Lehndorff (1909) reported 2 cases in which women after a series of syphilitic children gave birth to healthy children who in both cases were found to give a positive Wassermann reaction. Such cases must be regarded as cases of latent congenital syphilis, and it is in such cases that the use of the serum test should have a special value in leading to early treatment.

Bimfel (1909) examined the blood of 230 mothers. Each was examined at least twice during pregnancy and once during the puerperium. Among 21 cases, in which there was a suspicion of syphilis, a positive result was obtained in 13 and a negative result in 8 cases. Eleven of the women who reacted positively gave birth to 3 children with symptoms of syphilis (serum reaction positive), and 8 children without symptoms (serum reaction negative). Eight cases of secondary syphilis were examined, and a positive result both in mother and child obtained in 7. Of 9 cases of latent syphilis all gave a positive reaction; in 6 of the children a positive reaction was obtained, in 2 a partial reaction.

If it can be established that congenital syphilis is a frequent cause of idiocy, it is reasonable to hope that very great success will follow the application of therapeutic and prophylactic measures.

We now possess in the Wassermann reaction a means of diagnosis which enables us to detect syphilis in cases in which it cannot be recognised by any other method. Wassermann has suggested that the serum test should be applied to every woman who is admitted to a lying-in hospital. When we consider the numerous cases in which syphilis is quite unsuspected we must admit the value of his suggestion. If a positive reaction was obtained, treatment of the mother would be commenced at once, and treatment of the child might begin from the earliest possible time after its birth. It can hardly be doubted that benefit would follow from the wholesale adoption of such measures.

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ON THE MECHANISM OF COMPLEMENT FIXATION.

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(From the Bacteriological Laboratory of the Lister Institute.)

Any attempt to discuss the mechanism of the fixation of complement necessitates a brief reference to the well-known theories of Ehrlich and of Bordet.

The theory of Ehrlich assumes the existence of an amboceptor with two combining groups, one of which becomes attached to the antigen, while the other unites with the complement. The amboceptor is, in fact, supposed to form a link between the complement and the antigen. An haemolytic immune serum, for example, is supposed to contain an enormous number of amboceptors each of which possesses a cytophile group specific for the receptors of the red cell. After union has occurred between the receptors of the red cell and the cytophile group of the amboceptor, the other part of the amboceptor, the complementophile group is able to take up the complement. Bacteriolysis is thought to be brought about by the agency of a bacteriolytic amboceptor and the fixation of complement by a complement binding amboceptor. The amboceptor hypothesis has been supported by much indirect evidence but remains nevertheless a purely theoretical conception and does not in any case afford any real explanation of the experimental facts.

Bordet, on the other hand, considers that the complement is absorbed by the complex formed by the union of antigen and antibody. In 1901 Bordet and Gengou showed that complement is fixed by a mixture of an emulsion of *B. typhosus* with an antityphoid serum, and Gengou, in 1902, was able to fix complement in a mixture of a serum with its homologous antiserum. In a mixture of serum with its homologous antiserum, there occurs in the majority of cases a visible precipitate and the view was put forward that complement was taken up by the particles of the precipitate. This view was supported by Gay (1905), by Moreschi (1906) and by Moreschi and Pfeiffer (1906). It was, however, pointed out by Neisser and Sachs (1905), Muir and Martin (1906), Wassermann and Bruck (1905) and others that instances occurred when complement was fixed in mixtures in which no precipitate was visible and when no complement was bound in mixtures which produced a very considerable precipitate. The view was accordingly propounded that precipitation and complement fixation were separate and independent phenomena. The antibodies concerned in the formation of the precipitate were called precipitins and they were considered by many authors to be quite distinct from the antibodies which take part in complement fixation and which were held to be of the nature of amboceptors.

The literature dealing with the controversy is amply reviewed in an article by Sachs and Altmann (1909) and in an article by G. Meier (1909). For a brief consideration of the more important papers the reader may be referred to a paper in the *Proceedings of the Royal Society of Medicine* (Dean, 1911).

The chief evidence for the separate identity of the precipitins and the antibodies concerned in complement fixation rests on the numerous observations which show that complement is efficiently bound in a mixture of antigen and antibody in which no visible precipitation occurs. Such a mixture may be easily obtained by making a series of dilutions of antigen and adding to each tube a constant amount of antibody. In such a series of tubes complement fixation can as a rule be demonstrated for several tubes beyond the point where visible precipitation ceases. That is to say that for any quantity of antiserum a quantity of antigen may be found which when mixed with this quantity of antiserum produces no visible precipitate but which causes an efficient fixation of complement. Such an experiment is held to support the view that complement fixation and precipitation are separate and independent phenomena.

Our views as to how far the formation of a precipitate can be regarded as the essential cause of complement fixation must depend to a great extent on our knowledge of the formation and origin of the actual precipitate. The original theory assumes that the antibody which is called the precipitin, precipitates the antigen which is called the precipitinogen. The precipitin according to this view is an antibody which aggregates the particles of the antigen in the same fashion as an agglutinin is supposed to aggregate or agglutinate red cells or bacteria. Another view was expressed by Moreschi (1906) who stated that antigen and antibody united in various proportions to form a series of precipitates. Such precipitates would presumably contain variable proportions of the antigen and of the antibody according to the quantities of antigen and antibody present in the mixture. An entirely different explanation has been put forward by Welsh and Chapman (1906 to 1911) who have supported their conclusions by a series of exact quantitative experiments. These experiments show that in an ordinary precipitation experiment all or almost all of the precipitate is derived from the proteids of the antiserum. It is in fact the antigen (the so-called precipitinogen) which precipitates the proteids contained in the antiserum. Any quantity of an antiserum may be regarded as containing a definite content of precipitable material which can be precipitated by the addition of the homologous antigen, and the amount of precipitate which can be obtained from such a quantity of antiserum is a constant quantity which is independent within certain limits of the amount of antigen which is added.

If to a series of tubes containing a constant amount of antiserum there be added various amounts of antigen, several zones of precipitation may be arbitrarily differentiated. In the first place a great relative excess of antigen inhibits precipitation. In the next zone precipitation is very rapid. That is to say there is sufficient antigen present to quickly precipitate the precipitable material present in the antiserum. In these tubes large flocculi are rapidly formed and the process is soon complete. In the next zone, where a smaller amount of antigen is present, the process of precipitation is much slower. In these tubes there is sufficient antigen to precipitate all the precipitable substance of the antiserum provided that sufficient time is allowed. The entire process is slow and visible particles are only very slowly formed. In the last zone the tubes of which contain the smallest amounts of antigen, precipitation is very slow and incomplete. The quantity of antigen is insufficient to completely precipitate the precipitable content of the antiserum. These tubes show various grades of apparently homogeneous turbidity and opalescence and finally a point is reached where the amount of antigen present is insufficient to produce even a trace of opalescence.

If such an experiment be considered in the light of the theory of precipitation propounded by Welsh and Chapman many of the difficulties in connection with complement fixation disappear.

In the first place it can be shown (Dean, 1911) that the best complement fixation takes place when antigen and antibody are mixed in such proportions that precipitation takes place slowly. If there is sufficient antigen present to produce the rapid formation of large flocculi, little or no complement is fixed. It is during the earliest stage in the aggregation of the molecules which are to form the precipitate, that complement is fixed.

It was also shown that to obtain the maximum complement fixation in a mixture of serum and antiserum it is necessary that the complement should be present from the beginning of the reaction. If antigen is mixed with antibody and an interval of half an hour allowed (at 37° C.) before the complement is added, very much less fixation takes place. After a visible turbidity has made its appearance the capacity for fixing complement has to a large extent disappeared. In mixtures which contain very small amounts of antigen the process of precipitation is greatly prolonged. In such mixtures complement is efficiently fixed; for the early and incomplete stages of precipitation afford excellent conditions for the fixation of complement. In a mixture which contains so minute a quantity of antigen that no visible turbidity or opalescence appears it is probable that the earliest stages of the precipitation process do nevertheless take place and the formation of these minute invisible aggregations is the efficient cause of complement fixation. This appears to be the probable explanation of the fixation of complement in a mixture which contains too little antigen to produce a visible turbidity.

In carrying out a precipitation test it is possible to dilute the antigen to an enormous extent, frequently to 1 in 50,000 or 100,000, in exceptional cases even to 1 in 1,000,000. It is, however, customary to use a relatively large amount of the antiserum which is commonly employed in a dilution of 1 in 2 to 1 in 10. If a smaller quantity of antiserum be chosen, for instance a 1 in 100 dilution, little or no precipitate will be produced. The reason for this is apparent from the experiments of Welsh and Chapman, for in such cases there is not sufficient antiserum present to produce an appreciable precipitate. In complement fixation experiments on the other hand relatively small quantities of the antiserum can be employed. A good antiserum can be always used in a dilution of 1 in 100, often in a dilution of 1 in 500 or 1 in 1000. If, however, a cubic centimetre of a 1 in 500 dilution of antiserum be mixed with an equal quantity of a suitable dilution of the homologous antigen, not even a trace of a precipitate can as a rule be detected. Such a mixture contains far too little of the precipitable substance of the antiserum for the formation of a visible precipitate. Nevertheless such a mixture may serve admirably for complement fixation. Such an experiment can easily be performed with any good antiserum and the result at first sight appears to afford evidence for the view that precipitation and complement fixation are separate and independent phenomena.

The experiments which are to be described are concerned with an attempt to explain the fixation of complement in mixtures which contain a quantity of antiserum insufficient to produce a visible precipitate. The fixation of complement in mixtures which contain a quantity of antigen insufficient to form a precipitate has already been considered in a paper published in the *Proceedings of the Royal Society of Medicine,* December 1911.

Before proceeding further, reference must be made to certain observations which have been made on the phenomenon of agglutination. Muir and Browning (1906) observed that if normal ox serum was added to a mixture of a suspension of ox corpuscles and the homologous antiserum (rabbit *v.* ox) a very intense agglutination of the corpuscles was produced. The fresh serum was found to greatly augment the agglutinative action of the antiserum on the corpuscles. They also stated that if the ox serum was heated for an hour at 55° C. it lost the property of augmenting agglutination. In the same year Bordet and Gay (1906) published the results of very similar experiments. Bordet and Gay found that the property of increasing agglutination which was lost when ox serum was heated at 56° C. could be restored by adding a little of the fresh serum of another animal. They concluded that there exists in ox serum a special substance which resists a temperature of 56° C. and this substance they called "Colloide du bœuf." The substance was subsequently named conglutinin and the agglutination produced by the joint action of antiserum and ox serum has been called conglutination. Bordet and Streng (1909) subjected ox serum, after it had been heated to 56° C., to dialysis. They found that the fraction which remained in solution favoured haemolysis, while the precipitate if re-dissolved in normal saline solution favoured agglutination. Streng (1909) in another communication adopted the conglutination method as a means of differentiating bacteria. By the addition of ox serum and complement he obtained marked agglutination of bacteria with a dilution of homologous antiserum, which by itself was too weak to produce any trace of agglutination. Streng stated that conglutinin could be separated from agglutinin by dialysis. The agglutinin, under these conditions, remained in solution, while the conglutinin was precipitated with the globulin fraction. Barikine (1910) effected a similar separation of agglutinin and conglutinin by saturating ox serum with carbon dioxide. As in the dialysis experiment, the conglutinin was precipitated with the euglobulin fraction, and the agglutinin remained in solution. Barikine also stated that the flocculi of a precipitate, formed in a mixture of antigen and antibody, could be conglutinated by the addition of fresh serum (complement) and heated ox serum (conglutinin). Dean (1911) precipitated the euglobulin from fresh guinea-pig serum, collected the precipitate and re-dissolved it in 0·85 % sodium chloride solution. The solution of guinea-pig globulin was found to greatly increase the agglutination of sheep red cells by the homologous antiserum (rabbit *v.* sheep) and the agglutination of typhoid bacteria by antityphoid serum. If the antiserum was diluted to such an extent that it was no longer able to produce agglutination, marked clumping of the bacteria or cells could be produced by the addition of the guinea-pig globulin solution. It was, of course, shown by appropriate controls that the globulin acting by itself had no power to produce agglutination. If a mixture of sheep red cells, antiserum and guinea-pig globulin was incubated for an hour and then centrifugalised, it was found that the red cells had removed the agglutinating substance (? conglutinin) and the supernatant fluid had no power to produce agglutination if added to a second mixture of red cells and antiserum. The globulin was apparently adsorbed by the sensitised red cells. In a subsequent experiment red cells were lysed with distilled water and the solution was filtered to free it from stromata. In a mixture containing the soluble products of the red cells, antiserum and globulin solution, a fine flocculent precipitate formed. Control mixtures were also prepared: (*a*) red cell solution with antiserum, (*b*) red cell solution with globulin solution. In the control mixtures no precipitate formed. A similar phenomenon was observed when the guinea-pig globulin solution was added to a mixture of horse serum (antigen) with its homologous antiserum (rabbit *v.* horse), the antiserum being in this case used in a dilution of 1 in 100: this dilution was shown to be too weak to produce a precipitate when used in a simple mixture with the homologous antigen.

As a result of these experiments it was suggested that an essential part of the mechanism of agglutination was the precipitation of the globulin of the antiserum on the surface of the corpuscle or bacterium.

When the antiserum was diluted to such an extent that there was not sufficient globulin present for agglutination, its action could be reinforced by the addition of the globulin obtained from normal serum.

The results, moreover, appeared to have a distinct bearing on the mechanism of complement fixation. It was determined to prepare mixtures of antigen with antiserum so diluted that little or no precipitate was formed and to add to such mixtures fresh guinea-pig serum. The results of these experiments are recorded in the following pages.

Preparation of experimental material.

The materials used as antigens were normal human serum, normal horse serum, and watery extracts of *B. typhosus* perpared by a method described in the *Proceedings of the Royal Society of Medicine*, 1911, Vol. IV, p. 251.

The antisera were prepared by the intravenous injection of rabbits. The antityphoid sera were prepared by the injection of emulsions of killed bacteria in normal saline.

The haemolytic system consisted of a 1 in 20 emulsion of washed sheep corpuscles in 0·85 % sodium chloride solution. The haemolytic serum was prepared in the usual manner by the intravenous injection of rabbits with small quantities of washed corpuscles.

For the preparation of the mid-piece and end-piece fractions of the guinea-pig complement the carbon dioxide method of Liefmann (1909) was employed. The details of the method used may be found in a paper by Ledingham and Dean in the *Journal of Hygiene*, Vol. XII, No. 2, p. 154.

The results obtained by adding fresh guinea-pig serum (complement) to a mixture of antigen and antibody.

The materials used in this experiment were normal human serum and antihuman serum obtained by injecting a rabbit with human serum. Both sera were inactivated in the usual way by heating for half an hour at 56° C.

The first part of the table illustrates the results of a precipitation experiment. Eleven dilutions of the antigen were prepared; the dilutions varied from 1 in 40 to 1 in 40,960. The bulk of the diluted antigen was in each case 1 c.c. Eight sets of antigen dilutions were prepared. To every tube of four of these sets was added 1 c.c. of a

Scheme to illustrate the diagrams used in the Tables.

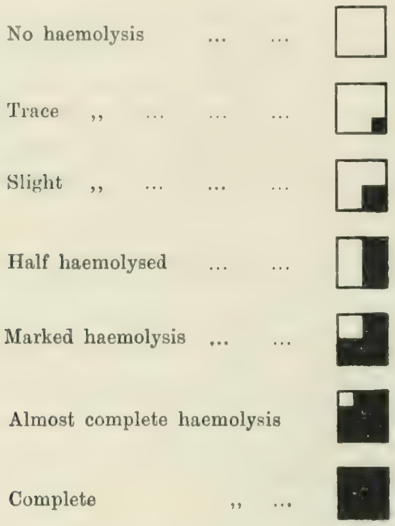

TABLE I. (*Part I.*) *Precipitation Experiment.*

1 c.c. human serum (antigen) diluted	+1 c.c. antiserum diluted 1 in 40		+1 c.c. antiserum diluted 1 in 80		+1 c.c. antiserum diluted 1 in 160		+1 c.c. saline solution	
	+1 c.c. complement 1 in 5	+1 c.c. saline solution	+1 c.c. complement 1 in 5	+1 c.c. saline solution	+1 c.c. complement 1 in 5	+1 c.c. saline solution	+1 c.c. complement 1 in 5	+1 c.c. saline soln.
(1) 1 in 40	+ + +	+ + +	+ Cloudy	Cloudy	Clear	Clear	Clear	Clear
(2) 1 in 80	+ + + +	+ + + +	+ Cloudy	+ Cloudy	Slightly cloudy	,,	,,	,,
(3) 1 in 160	+ + + +	+ + + +	+ + +	+ +	+ Cloudy	,,	,,	,,
(4) 1 in 320	+ + + +	+ + + +	+ + +	+ +	+	,,	,,	,,
(5) 1 in 640	+ + + +	+ + + +	+ + +	+ +	+	Trace opalescence	,,	,,
(6) 1 in 1280	+ + + +	+ + +	+ + +	+	+	Clear	,,	,,
(7) 1 in 2560	+ + +	+ Cloudy	+ +	Cloudy	+	,,	,,	,,
(8) 1 in 5120	+ + +	Cloudy	+ Cloudy	Opalescent	+	,,	,,	,,
(9) 1 in 10240	+ +	Trace opalescence	+ Cloudy	Clear	+	,,	,,	,,
(10) 1 in 20480	+	Clear	Cloudy	,,	Cloudy	,,	,,	,,
(11) 1 in 40960	Opalescent	,,	Opalescent	,,	Clear	,,	,,	,,
(12) 1 c.c. saline soln.	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear

TABLE I. *(Part II.) Complement Fixation Experiment.*

1 c.c. human serum (antigen) diluted	+1 c.c. antiserum			+1 c.c. saline solution
	Diluted 1 in 40	Diluted 1 in 80	Diluted 1 in 160	
(1) 1 in 40	■	■	■	■
(2) 1 in 80	◲	▟	■	■
(3) 1 in 160	□	◲	◨	■
(4) 1 in 320	□	□	◲	■
(5) 1 in 640	□	□	◲	■
(6) 1 in 1280	□	□	◲	■
(7) 1 in 2560	◲	□	◲	■
(8) 1 in 5120	◲	□	◲	■
(9) 1 in 10240	▟	◲	◲	■
(10) 1 in 20480	■	▟	▟	■
(11) 1 in 40960	■	■	■	■
(12) 1 c.c. saline solution	■	■	■	■

1 in 5 dilution of fresh guinea-pig serum. To each tube of the other four sets was added 1 c.c. of saline solution. Three dilutions of antiserum were prepared, namely 1 in 40, 1 in 80 and 1 in 160. One c.c. of each dilution of the antigen was then added to every tube of two sets, the one set containing complement in each case and the other set containing only antigen and saline. To the last two sets no antiserum was added, but each tube received 1 c.c. of normal saline. The tubes

were incubated for five hours at a temperature of 37° C. and were then placed overnight in a room at a temperature of 8° C. The results were then read and recorded.

The crosses in the table indicate a deposit of precipitate and a clear supernatant fluid. The number of crosses represent very roughly the relative amount of precipitate in each tube. In some tubes precipitation was evidenced by a uniform turbidity which had not settled to form a deposit. In some cases some of the precipitate had deposited while the rest remained in suspension.

The experiment shows the amount of precipitate formed by various mixtures of antigen and antibody. These are represented in the right-hand columns of each of the larger divisions. The left-hand columns show the influence of the addition of the fresh serum of the guinea-pig to these mixtures.

It will be seen that precipitation was much more evident in the sets to which guinea-pig serum was added. The tubes represented by the first two columns of the table contained 1 c.c. of a 1 in 40 dilution, that is to say 0·025 of the antiserum.

In the tubes which contained the larger quantities of antigen (1 in 40 to 1 in 1280) there was no appreciable difference in the precipitation between the two sets: in both cases a flocculent precipitate had completely separated from a clear supernatant fluid.

In the tubes which contained the smaller amounts of antigen there was a distinct difference between the two sets.

The tubes containing the complement (except Tube 11) showed definite deposits and a clear supernatant fluid while the corresponding tubes which had received no complement showed various stages of uniform turbidity or opalescence. In the tubes which contained complement the precipitation process had proceeded to the stage of formation of visible flocculi which had fallen to the bottom of the tube. In the tubes containing no complement the particles were too small to be individually visible and remained suspended in the fluid in the form of an apparently homogeneous turbidity.

In the tubes which contained half the amount of antiserum, namely, 1 c.c. of a 1 in 80 dilution the difference between the tubes which did contain complement and those which did not, was much more marked. In the case of the mixtures which contained the smaller quantities of antigen the tubes containing complement showed a deposit while those which contained no complement showed a uniform turbidity. In the case of the tubes which contained the larger quantities of antigen both sets showed deposits but the deposits in the tubes containing complement were definitely larger than the deposits in the control tubes without complement. When the amount of antiserum was reduced still further to 1 c.c. of a 1 in 160 dilution (= 0·00625 c.c. of antiserum) a still greater difference was apparent. In the control set to which no complement was added there was no trace of precipitation except in Tube 5 in which a rather doubtful trace of opalescence was recorded. In the majority of the tubes to which complement had been added a small but perfectly definite precipitate was present. In the last two columns and in the bottom line (No. 12) the necessary control tubes are represented. The controls containing the human serum (antigen) and the antiserum in all the dilutions employed remained perfectly clear during the period of the experiment (20 hours). No turbidity or precipitate was produced by any of the mixtures of antiserum and complement or by any of the mixtures of antigen and complement.

The second part of the table represents the result of a complement fixation experiment in which the same dilutions of antigen and antiserum were employed as in the precipitation experiment.

Four sets of antigen dilutions were prepared and the bulk of the antigen dilution in each tube was 0·5 c.c. To each tube was then added 0·5 c.c. of a 1 in 10 dilution of fresh guinea-pig serum. To each of the tubes of the first column was then added 0·5 c.c. of a 1 in 40 dilution of the antiserum, to each of the tubes of the second column 0·5 c.c. of a 1 in 80 dilution of the antiserum, to each of the tubes of the third column 0·5 c.c. of a 1 in 160 dilution of the antiserum. The tubes of the fourth column constituted the antigen controls and received 0·5 c.c. of saline solution. The tubes numbered 12 in each column contained the same dilution of antiserum as the other tubes of the column but no antigen. All the tubes were then incubated at a temperature of 37° C. for one hour. At the end of the hour all the tubes received 0·5 c.c. of a 1 in 400 dilution of haemolytic serum (rabbit *v.* sheep) and 0·5 c.c. of a 1 in 20 suspension of washed sheep corpuscles. All the tubes were then incubated for a further period of two hours.

On looking at Table I it will be seen that the increase in precipitation in the sets containing guinea-pig serum occurred in those tubes which contained the relative proportions of antigen and antiserum which were shown in the second part of the table to be suitable for the fixation of complement. That is to say, the zones of extra-precipitation corresponded fairly closely with the zones of complement fixation. In the case of the tubes which contained an antiserum dilution of 1 in 160 practically no precipitation occurred in the tubes containing the mixture of antigen and antiserum while excellent complement fixation was obtained. A result such as this is frequently used as an argument in favour of the view that precipitation is separate and distinct from complement fixation. The table, however, shows that if fresh guinea-pig serum (complement) is added to such mixtures of antigen and antiserum a precipitate does actually form. If guinea-pig serum is added to a mixture of antigen and antiserum which will bind complement, a precipitate is formed even if no precipitate is visible in a simple mixture of the antigen and antibody. The fact that no precipitate is visible in the simple mixture of antigen and antibody is no evidence that complement fixation is independent of the formation of a precipitate.

It is necessary to state that the smaller precipitates shown in the table did not become visible during the first few hours after the commencement of the experiment. The precipitates recorded in the table were observed and recorded 20 hours after the antigen, antiserum and guinea-pig serum had been mixed. This observation is consistent with results previously published (Dean, 1911) which showed that mixtures of antigen and antibody in which the formation of a precipitate was slow were favourable to the fixation of complement.

The explanation of the extra precipitate caused by the presence of complement in a mixture of antigen and antibody is a matter of some difficulty. In mixtures which contain a large amount of antiserum and in which a bulky precipitate is formed, the addition of guinea-pig serum makes no appreciable difference to the amount of the precipitate. When, however, the amount of the antiserum in the mixture is very small the quantity of precipitable proteid (antiserum) is so small that the resulting precipitate is not visible. If to such a mixture there be added normal guinea-pig serum it is quite possible that the proteids of the guinea-pig serum are adsorbed by the minute particles of the specific precipitate. The particles of the precipitate are thus increased in size and become visible as a definite turbidity or precipitate. It is suggested that this adsorption of the proteids of the guinea-pig serum by the minute particles of a precipitate is the essential cause of the fixation of complement.

Another explanation might be afforded by supposing that the addition of complement did not cause any increase in the bulk of the precipitate, but merely acted by agglutinating or conglutinating the particles of the precipitate and in this fashion making the precipitate more easily visible. Barikine (1910) in fact explained his experiments in this way. After a consideration, however, of the experiment recorded in Table I and of numerous other similar experiments the conglutination explanation did not appear to account for the facts. It seemed for instance very doubtful if the quite definite precipitates obtained by adding guinea-pig serum to a 1 in 160 dilution of the antiserum (Table I) could be explained by a mere agglutination of an already formed precipitate. Further evidence on this point is afforded by the experiment recorded in Table III.

The effect of adding fresh guinea-pig serum to mixtures of an extract of B. typhosus *with antityphoid serum.*

Many experiments of the type of that recorded in Table I were performed and a similar result was in all cases obtained. In the experiment recorded in Table II the antigen was a distilled water extract of *B. typhosus*. The antiserum had been obtained by the intravenous injection of a rabbit with emulsions of killed typhoid bacteria. The experiment was carried out in essentially the same manner as that recorded in Table I. In the complement fixation experiment, three different quantities of complement were used in order to provide a roughly quantitative measure of the complement-binding power of the various mixtures employed. It will be seen that precipitation was far more marked in the set of tubes to which the guinea-pig serum was added. Similar results were obtained with other antigen-antibody systems.

The effect of adding various quantities of guinea-pig serum to a mixture of antigen and antibody.

In this experiment tubes were used the lower portions of which were narrow and calibrated in order that the amount of precipitate formed could be measured. Only one quantity of antigen was employed (3 c.c. of a 1 in 20 dilution of typhoid extract) and one quantity of the antiserum (3 c.c. of a 1 in 20 dilution of antityphoid serum). The amount of precipitate formed by the simple mixture of the antigen and antibody is shown in the second column, Tube 8. The actual bulk of the precipitate was 0·01 c.c.

The table shows that the addition of guinea-pig serum to this mixture caused a very marked increase in the bulk of the precipitate.

TABLE II.

Precipitation Experiment.

3 c.c. extract of *B. typhosus* diluted	+3 c.c. antityphoid serum (rabbit) diluted 1 in 20		
	+3 c.c. of fresh guinea-pig serum diluted 1 in 5	+3 c.c. of saline solution	+3 c.c. saline solution +3 c.c. guinea pig serum diluted 1 in 5
(1) 1 in 10	Flocculent precipitate — turbid fluid	Slight turbidity	Clear
(2) 1 in 20	Large flocculent precipitate—clear fluid	,,	,,
(3) 1 in 40	,, ,,	,,	,,
(4) 1 in 80	Flocculent precipitate—clear fluid	,,	,,
(5) 1 in 160	Small particles —clear fluid	Opalescent	,,
(6) 1 in 320	Opalescent	Clear	,,
(7) 1 in 640	? Trace opalescence	,,	,,
(8) 3 c.c. saline solution	Clear	,,	,,

Precipitation. Result after 24 hours.

The middle column shows the precipitation produced by mixing various quantities of typhoid extract with a constant quantity of antityphoid serum. The left-hand column shows the result obtained when fresh guinea-pig serum is added to these various mixtures of extract and serum. The third column shows that no precipitate is produced when normal guinea-pig serum is mixed with typhoid extract. Tube 8 in the left-hand column shows that no precipitate is produced by the mixture of the antityphoid serum with normal guinea-pig serum.

Complement Fixation Experiment.

0·5 c.c. extract of *B. typhosus* diluted	+0·5 c.c. antityphoid serum diluted 1 in 20			Extract controls +0·5 c.c. saline solution, 0·5 c.c. complmt. 1 in 10
	0·5 c.c. complement 2 in 5	0·5 c.c. complement 1 in 5	0·5 c.c. complement 1 in 10	
(1) 1 in 10	◲	□	□	▟
(2) 1 in 20	◲	□	□	■
(3) 1 in 40	◲	□	□	■
(4) 1 in 80	▟	□	□	■
(5) 1 in 160	■	◲	□	■
(6) 1 in 320	■	■	◲	■
(7) 1 in 640	■	■	▟	■
(8) 0·5 c.c. saline soln. (serum control)	■	■	■	

Complement Fixation.

This experiment shows the amount of complement fixed by mixtures of typhoid extract and antiserum identical with those used in the precipitation experiments. The dilutions of extract, antiserum and fresh guinea-pig serum (complement) were all used in a bulk of 0·5 c.c. After one hour's incubation each tube received 0·5 c.c. of a 1 in 20 suspension of sheep corpuscles and 0·5 c.c. of a 1 in 300 dilution of haemolytic serum (rabbit *v.* sheep). The results were read after a second period of incubation lasting two hours.

In the tubes containing the large quantities of guinea-pig serum the bulk of the precipitate was twice and three times as great as the precipitate produced in the simple mixture of antigen and antibody. It is difficult to escape the conclusion that the larger precipitates were partly derived from the proteids of the guinea-pig serum.

TABLE III.

To illustrate result obtained by adding various amounts of fresh guinea-pig serum (complement) to a mixture of typhoid extract and antityphoid serum.

		+3 c.c. antityphoid serum 1 in 20 +3 c.c. typhoid extract 1 in 20		+3 c.c. saline solution +3 c.c. typhoid extract 1 in 20	+3 c.c. antityphoid serum +3 c.c. saline solution	+6 c.c. saline solution
	3 c.c. of guinea-pig serum	After 6 hours	After 24 hrs. centrifugalised deposit	After 24 hours	After 24 hours	After 24 hours
1	Undiluted	Large flocculi	3	Clear	Clear	Clear
	Diluted					
2	1 in 2½	,, ,,	3	,,	,,	,,
3	1 in 5	,, ,,	2	,,	,,	,,
4	1 in 10	Small particles	1	,,	,,	,,
5	1 in 20	Turbid	1	,,	,,	,,
6	1 in 40	,,	1	,,	,,	,,
7	1 in 80	,,	1	,,	,,	,,
8	Control 3 c.c. saline solution	,,	1	,,	,,	,,

All the tubes were incubated for 6 hours at 37° C. The tubes were then placed in a cool room at 8° C. After 24 hours the tubes in the first column were centrifugalised and the deposits observed. All the control tubes remained absolutely clear.

The effect of adding normal rabbit serum, normal ox serum and heated guinea-pig serum to mixtures of antigen and antibody.

The controls employed in all these experiments gave perfectly satisfactory results. In no case did either antigen or antibody in the dilution employed cause any precipitation when mixed with guinea-pig serum. Many of the experiments were, however, repeated, and fresh normal rabbit serum was substituted for the normal guinea-pig serum. In these experiments the possibility that the results obtained were due to the action of normal precipitins in the rabbit or guinea-pig serum could be definitely excluded. The results of the experiments were similar to those obtained by the use of normal guinea-pig serum.

In view of the remarkable "conglutinative" property which ox serum has been shown to possess it was decided to try normal ox serum in place of normal guinea-pig serum. The addition of normal ox serum to a mixture of typhoid extract and antityphoid serum produced an increase in the precipitation. This increase was about the same as that produced by the addition of an equal quantity of normal guinea-pig serum.

In another experiment some normal guinea-pig serum was heated for half an hour at 56° C. A series of dilutions of the heated and of the unheated guinea-pig serum were then added to a mixture of typhoid extract and antityphoid serum. This experiment was carried out in the same fashion as the experiment recorded in Table III. There was very little difference between the results obtained with the fresh guinea-pig serum and those obtained with the heated guinea-pig serum. The increased precipitation was a trifle more marked in the tubes which contained the fresh serum. This shows that the action of the normal serum in these experiments does not depend on the presence of complement.

The part played by the two fractions of the complement in fixation experiments.

Before proceeding further it is necessary to refer to the experiments of Ferrata and others who have shown that complement must be no longer regarded as a single substance.

Ferrata (1907) dialysed fresh guinea-pig serum against distilled water. He then separated the precipitated euglobulin and re-dissolved it in saline solution. To the residue which remained in solution he added sufficient sodium chloride to make the solution isotonic with blood serum. He thus obtained two fractions, (1) a globulin fraction, and (2) a so-called albumen fraction. He found that neither fraction could act as complement but that excellent complementing could be obtained by adding a mixture of the two fractions to corpuscles and haemolytic serum. Brand (1907) repeated these experiments and called the fraction which was precipitated with the euglobulin the mid-piece (Mittelstück) and the fraction which remained in solution the end-piece (Endstück). These names are convenient and are now in general use. Ferrata's method for separating the fractions was, however, found to be difficult and somewhat unreliable and two other methods have been devised. Liefmann (1909) diluted one part of fresh guinea-pig serum with nine parts of distilled water and saturated the mixture with carbon dioxide gas. The precipitated euglobulin is separated in a centrifuge, washed with distilled water and re-dissolved in salt solution

This constitutes the mid-piece solution. To the supernatant fluid is added enough sodium chloride to make the solution isotonic with blood serum. This constitutes the end-piece solution. Sachs and Altmann (1909) effected a separation of the two fractions by using very dilute hydrochloric acid.

It should be noticed that all these methods consist in producing a precipitate of the euglobulin of the solution. The mid-piece of the complement is found to be precipitated with the euglobulin fraction of the serum.

The behaviour of the two fractions of the complement when fresh guinea-pig serum (the whole complement) is added to a mixture of antigen and antibody has been investigated by several authors.

Skwirsky (1910) stated that in the Wassermann reaction the mid-piece fraction was bound while the end-piece remained absolutely unbound. The same result was obtained when complement was added to a mixture of tuberculin and antituberculin or to a mixture of horse serum and antihorse serum (rabbit *v.* horse). It was also shown in the case of certain sera, which had the property of spontaneously binding complement, that only the mid-piece fraction was taken up. In haemolysis experiments on the other hand both fractions of the complement disappeared. Skwirsky also experimented with suspensions of kaolin which he found to take up both fractions of the complement.

Henderson Smith (1910) found that in precipitin reactions and in Wassermann reactions the mid-piece only was fixed. Spontaneously binding serums and bacterial extracts were also found to bind mid-piece only. Sachs and Bolkowska (1910) mixed red corpuscles, haemolytic serum and complement, and allowed the mixture to stand at 0° C. When a small quantity of the antiserum was used no complement was bound, when the amount of antiserum in the mixture was increased the mid-piece alone was taken up. When very large quantities of antiserum were employed both fractions of the complement were fixed and haemolysis followed. Amako (1910) found that in specific reactions between antigen and antibody the mid-piece was chiefly fixed while the end-piece remained free in solution. Occasionally partial binding of the end-piece occurred. Suspensions of kaolin, yeast cells and oxide of iron were found to bind both fractions of the complement. In the Wassermann reaction the mid-piece only was fixed. Amako concluded that the Wassermann reaction must for this reason be regarded as a specific reaction between an antigen and an antibody. Gengou (1911) found that in a suitably arranged experiment sensitised red corpuscles, sensitised cholera vibrios and specific precipitates fixed the whole of the mid-piece, while part at least of the end-piece remained free. A precisely similar result was obtained with an emulsion of barium sulphate. Gengou concludes that no distinction need be drawn between the absorption of complement by a barium sulphate precipitate and the absorption of complement which occurs in a complement fixation or in a haemolytic experiment.

From these experiments of Gengou it appears that there is no essential difference between specific and non-specific complement fixation. If complement is added to any complement binding substance or mixture of substances it is found that the mid-piece is more easily bound than the end-piece. If the mixture has only slight complement binding properties some or all of the mid-piece is fixed while the end-piece remains free. If the mixture has stronger complement binding properties all the mid-piece is bound and some of the end-piece. Very strong mixtures may bind the whole of both fractions.

We are now in a position to consider the various conditions under which complement is fixed. When fresh guinea-pig serum is dialysed against distilled water, when it is diluted with distilled water and saturated with carbon dioxide, when it is acidified with weak hydrochloric acid, the particles of euglobulin of the serum are aggregated and precipitated and when this occurs the mid-piece of the complement is fixed. The mid-piece of the complement is, in fact, taken up by the precipitate of euglobulin and if the precipitate is re-dissolved or re-suspended in saline the presence of the mid-piece can be demonstrated[1]. Now it has been shown in the experiments already described (Tables I, II and III) that when fresh guinea-pig serum is added to a suitable mixture of antigen and antibody an increase of precipitation occurs. The results of these experiments make it appear probable that under these conditions a part of the precipitate is actually derived from the proteids of the guinea-pig serum. That is to say, the reaction between antigen and antibody results in a partial precipitation of the proteids of the guinea-pig serum and the mid-piece of the complement is taken up by this precipitate in the same fashion as it is taken up by the precipitate of euglobulin produced by the action of carbon dioxide or weak hydrochloric acid.

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[1] These methods for separating the two fractions of the complement are successful because the mid-piece is much more readily taken up by the precipitate than the end-piece. In carrying out such a separation of the two fractions the experimental conditions are arranged to ensure that the mid-piece is fixed while the end-piece remains as far as possible in solution. The difference is, however, in this case also a quantitative one, for under suitable conditions it can be shown that the end-piece is also, though far less readily, fixed by a suspension of euglobulin.

A series of dilutions of a suspension of euglobulin are added to a constant quantity of end-piece. The various tubes are then incubated at 37° C. for one hour at the end of which time a mixture of red corpuscles, haemolytic serum and mid-piece (persensitised corpuscles) are added to each tube. The tubes are then again incubated and the amount of lysis in each tube is recorded. Such an experiment shows that euglobulin suspended in salt solution has distinct power of taking up end-piece. The fact that the end-piece fraction is taken up by a suspension of euglobulin in salt solution is the probable explanation of the so-called Brand phenomenon. Further experiments on this point may be found in the *Journal of Hygiene*, Vol. XII, No. 2, p. 183 (Ledingham and Dean).

The next step was to show that it is the euglobulin fraction of guinea-pig serum which is precipitated by the mixture of antigen and antiserum. That this is the case will be seen from the results recorded in Table IV.

The effect obtained by adding a solution of euglobulin to a mixture of typhoid extract and antityphoid serum.

The euglobulin preparations used in this experiment were prepared by Liefmann's method. The greatest care was taken to obtain a perfectly clear solution of euglobulin. This euglobulin solution contained the mid-piece of the complement. It is described in the table as 1 in 10 mid-piece. This means that the euglobulin precipitate obtained from 1 c.c. of guinea-pig serum was re-dissolved in 10 c.c. of saline solution.

This experiment was performed in precisely the same manner as that recorded in Table I.

The first part of the table shows the result of adding the euglobulin solution to various mixtures of typhoid extract (antigen) and antityphoid serum (antibody). All these tubes were incubated for four hours at 37° C. and then allowed to stand overnight in a cool room at a temperature of 8° C. The results were then read and recorded. The relative amounts of the deposit are indicated by the number of crosses in the table. In the mixtures which contained the largest quantity of antiserum, 1 c.c. of a 1 in 10 dilution, the addition of the euglobulin solution produced no visible increase in the amount of the precipitate. In the mixtures which contained the smaller quantities of antiserum there was a very evident difference between the tubes which contained the euglobulin and the control tubes which did not. The zones of the precipitation corresponded fairly well with the zones of complement fixation shown in the second part of the table. In the

TABLE IV.

Precipitation Experiment.

1 c.c. of extract of *B. typhosus* diluted	+1 c.c. antityphoid serum diluted 1 in 10		+1 c.c. antityphoid serum diluted 1 in 40		+1 c.c. antityphoid serum diluted 1 in 160		+1 c.c. saline solution	
	+ 1 c.c. mid-piece 1 in 10	+1 c.c. saline solution	+1 c.c. mid-piece 1 in 10	+1 c.c. saline solution	+1 c.c. mid-piece 1 in 10	+1 c.c. saline solution	+1 c.c. mid-piece 1 in 10	+1 c.c. saline solution
(1) 1 in 2½	+ +	+ +	Clear	Clear	Clear	Clear	Clear	Clear
(2) 1 in 10	+ + + +	+ + + +	+	,,	,,	,,	,,	,,
(3) 1 in 40	+ + + +	+ + + +	+	Trace opalescence	Opalescence	,,	,,	,,
(4) 1 in 160	+ +	+ +	+	,,	Slight turbidity	,,	,,	,,
(5) 1 in 640	+	Clear	Opalescence	Clear	Trace opalescence	,,	,,	,,
(6) 1 c.c. saline soln.	Clear	,,	Clear	,,	Clear	,,	,,	,,

Complement Fixation Experiment.

0·5 c.c. extract of *B. typhosus* diluted	+0·5 c.c. antityphoid serum diluted 1 in 10	+0·5 c.c. antityphoid serum diluted 1 in 40	+0·5 c.c. antityphoid serum diluted 1 in 160	+0·5 c.c. saline solution
(1) 1 in 2½	□	□	▗	▗
(2) 1 in 10	□	▗	▟	■
(3) 1 in 40	□	□	■	■
(4) 1 in 160	□	□	◨	■
(5) 1 in 640	▗	▗	▟	■
(6) 0·5 c.c. saline soln.	■	■	■	

complement fixation experiment, the same dilutions of antigen and antibody were used as in the precipitation experiment. To every tube was added 0·5 c.c. of fresh guinea-pig serum diluted 1 in 10. After incubation for one hour at 37° C. each tube received 0·5 c.c. of a 1 in 20 suspension of sheep corpuscles and 0·5 c.c. of a 1 in 400 dilution of haemolytic serum. The results were read after a further period of incubation lasting two hours.

It will be seen that when a 1 in 160 dilution of antiserum was used no precipitate was formed in the simple mixtures of antigen and antibody, while the addition of this quantity of the homologous antigen produced definite fixation of complement. In the series of tubes which received the solution of euglobulin, definite turbidity occurred in three of the tubes.

This result was supported by the results of numerous similar experiments in which various antigens and antiserums were employed. In all these experiments the same method was employed. Precipitation and complement fixation experiments were carried out in which various quantities of the antigen were mixed with various quantities of the antiserum. In this way the dilution of antiserum was determined which just failed to produce visible precipitation but was nevertheless effective for the fixation of complement. To such a dilution of antiserum was then added a suitable dilution of the antigen together with the euglobulin preparation. In such a mixture definite precipitation was found to take place.

As a result of the reaction which takes place between antigen and antibody the particles of euglobulin of the guinea-pig serum are aggregated and precipitated. The mid-piece fraction of the complement is probably fixed or bound by this euglobulin precipitate in precisely the same manner as it is fixed by the euglobulin precipitate which is produced by the action of carbon dioxide or weak hydrochloric acid. The precipitation of the euglobulin of the guinea-pig serum is probably an essential part of the mechanism of complement fixation.

These experiments do not, of course, afford any evidence as to the mechanism by which the euglobulin of guinea-pig serum is precipitated by a suitable mixture of antigen and antiserum. It is possible that the euglobulin is adsorbed by minute and invisible particles of precipitate formed in the mixture of antigen and antibody. We have, however, at the present time no knowledge of the mechanism by which a precipitate is formed in a simple mixture of antigen and antibody, and such knowledge is probably essential to a complete understanding of the mechanism of complement fixation.

Is it possible to recover the mid-piece of the complement from the precipitate which occurs in a mixture of antigen, antibody and complement?

The experiments described in the previous paragraph suggest that the fixation of complement by a mixture of antigen and antibody is a reaction similar to the fixation of complement by the euglobulin precipitate produced by treating guinea-pig serum with carbon dioxide or weak hydrochloric acid. It was in fact shown that a suitable mixture of antigen and antibody does precipitate an euglobulin solution prepared from guinea-pig serum.

When complement is added to a suitable mixture of antigen and antibody we know that complement disappears. We are accustomed to test for this disappearance of complement by the addition of another antigen-antibody system, namely a mixture of corpuscles and haemolytic system. If the mixture after a period of incubation contains no free complement, no haemolysis takes place and we say that complement is fixed or bound. There are obviously two possibilities:

(1) The complement has been used up or destroyed.

(2) The complement has not been used up but has been rendered temporarily inactive and may by appropriate measures be recovered in an active state.

We know from the work of the authors recently quoted that it is chiefly the mid-piece of the complement which is bound by a mixture of a serum with its homologous antiserum, while the end-piece remains comparatively unaffected. If we add complement to a mixture of serum and antiserum, the mid-piece is taken up by the precipitate. Is the mid-piece used up or destroyed when it is bound or is it possible to recover it in an active state from the precipitate? If we observe the progress of haemolysis in an ordinary complement fixation experiment we must come to the conclusion that the mid-piece is not destroyed when it is bound. It is a common practice to add various quantities of an antiserum to a constant quantity of antigen. These various mixtures have, of course, various degrees of complement binding power. To all these tubes is then added a constant quantity of complement. The mixture is then incubated for an hour and blood and haemolytic serum are added to each tube. If we now watch the progress of haemolysis, we find that the tubes containing the antigen and antiserum controls are the first to dissolve. We may then remove the experiment from the incubator and record the results. If however we decide to continue the experiment we shall find that certain tubes which at first showed no trace of haemolysis, gradually begin to dissolve. In these tubes the mid-piece has been dissociated from the original antigen-antibody complex and is utilised by the corpuscles and haemolytic system. As the experiment is continued ,the weaker mixtures of the original antiserum and antigen give up more or less of their complement to the haemolytic system. After one or two hours very little further haemolysis takes place; that is to say, a point is reached when the weaker mixtures have given up their complement to the haemolytic system, while the mixtures of antigen and antibody, which had greater complement binding power, are able to retain the complement.

A more evident proof that the mid-piece is not destroyed may be obtained by adding various amounts of the haemolytic serum to a series of tubes each containing the same quantity of antigen, antiserum and complement. In a suitably arranged experiment it can be shown that the amount of complement fixation depends under these conditions on the quantity of the haemolytic serum which is added. There may, for example, be no haemolysis in the tube to which a 1 in 800 dilution of the haemolytic serum was added. That is to say all the complement has been fixed. While in a tube which contains precisely the same quantities of antigen, antiserum and complement, but to which a 1 in 200 dilution of the haemolytic serum has been added, a marked degree of haemolysis may take place. In both cases the complement was fixed but the stronger dose of haemolytic serum has been able to dissociate a certain amount of the complement from the original antigen-antibody complex.

It is obvious that the mid-piece is not destroyed when it is bound by a mixture of antigen and antibody.

If the precipitate obtained by the action of carbon dioxide or weak hydrochloric acid is collected, washed and re-dissolved, it is possible to show that the resulting solution does contain the active mid-piece fraction of the complement. Is it possible to demonstrate the presence of active mid-piece in the precipitate resulting from the mixture of antigen, antibody and complement?

Numerous experiments with this object were carried out, the results being at first extremely unsatisfactory. A satisfactory result was eventually obtained by the use of the following method:

In a test tube was placed 1 c.c. of fresh guinea-pig serum and 7 c.c. of saline solution. A second test tube contained a 1 in 100 dilution of normal human serum (the antigen), while a third test tube contained undiluted antiserum (rabbit *v.* man). All three tubes were allowed to stand for one hour in the cold room at a temperature of 0 C. When all the ingredients were quite cold, 1 c.c. of the 1 in 100 dilution of the human serum and 1 c.c. of the undiluted antiserum were added to the tube containing 8 c.c. of diluted complement. The tube was then well shaken. The tube contained a bulk of 10 c.c. The concentration of the human serum (antigen) was 1 in 1000, of the fresh guinea-pig serum (complement) 1 in 10 and of the rabbit *v.* man serum (antiserum) 1 in

10. The mixture was allowed to remain for three hours at 0° C. during which time it was occasionally shaken. After three hours a definite deposit of precipitate had formed and the mixture was then centrifugalised in the cold room. The supernatant fluid which was quite clear was poured off and the precipitate was suspended in cold distilled water and again centrifugalised. This washing process was repeated and the precipitate was finally re-suspended in cold 0·85 % saline solution. A uniformly turbid emulsion was obtained.

The materials used in the subsequent experiment (Table V) were:

(1) The suspension of the precipitate in normal saline solution.

(2) An ordinary mid-piece preparation made from guinea-pig serum by Liefmann's method.

(3) A 1 in 20 suspension of washed sheep red corpuscles.

(4) A 1 in 200 dilution of a haemolytic serum (rabbit *v.* sheep cells) the titre of which was 1 in 1200.

All the above were cooled to 0° C. before mixing.

(5) The supernatant fluid from the precipitation experiment.

(6) An ordinary end-piece preparation made from guinea-pig serum by Liefmann's method.

TABLE V.

	Emulsion of precipitate	Mid-piece preparation (Liefmann's method)	Suspension of sheep corpuscles (1 in 20)	Haemolytic serum diluted 1 in 200	Saline solution		Supernatant fluid	End-piece preparation (Liefmann's method)	Saline solution	Result after 2 hours at 37° C.
(1)	1 c.c.	—	0·5 c.c.	0·5 c.c.	—	Interval of 1 hour at 0° C.	1 c.c.	—	—	Slight haemolysis.
(2)	1 c.c.	—	0·5 c.c.	0·5 c.c.	—		—	1 c.c.	—	Complete haemolysis.
(3)	1 c.c.	—	0·5 c.c.	0·5 c.c.	—		—	—	1 c.c.	No haemolysis.
(4)	—	1 c.c.	0·5 c.c.	0·5 c.c.	—		1 c.c.	—	—	Almost complete haemolysis.
(5)	—	1 c.c.	0·5 c.c.	0·5 c.c.	—		—	1 c.c.	—	Complete haemolysis.
(6)	—	1 c.c.	0·5 c.c.	0·5 c.c.	—		—	—	1 c.c.	No haemolysis.
(7)	—	—	0·5 c.c.	0·5 c.c.	1 c.c.		1 c.c.	—	—	Trace haemolysis.
(8)	—	—	0·5 c.c.	0·5 c.c.	1 c.c.		—	1 c.c.	—	No haemolysis.
(9)	—	—	0·5 c.c.	0·5 c.c.	1 c.c.		—	—	1 c.c.	No haemolysis.

The mixtures shown in the table were then prepared. The corpuscles and haemolytic serum were mixed with the mid-piece preparation or the suspension of washed precipitate at a temperature of 0° C. and allowed to remain at this temperature for one hour. All the tubes were then removed from the cold room and supernatant fluid, end-piece or saline solution was added to each tube as shown in the table. All the tubes were then incubated at 37° C. for two hours. The degree of haemolysis was then observed and recorded.

Tube 3 shows that the precipitate did not contain the whole complement. No haemolysis was observed in the tube which contained corpuscles, haemolysin and precipitate. But the precipitate did contain the mid-piece fraction of the complement in an active state for complete haemolysis occurred in Tube 2. In this tube the mid-piece present in the precipitate was supplemented by the addition of end-piece prepared by Liefmann's method. The supernatant fluid contained a fair amount of end-piece and a trace of mid-piece. A trace of haemolysis was observed in the tube which contained corpuscles, haemolysin and supernatant fluid. The supernatant fluid contained sufficient end-piece to produce almost complete haemolysis when added to corpuscles persensitised with haemolysin and mid-piece prepared by Liefmann's method. Tube 1 shows that slight haemolysis occurred when the supernatant fluid was added to a mixture of corpuscles, haemolysin and suspended precipitate. In this case the end-piece present in the supernatant fluid was probably taken up by the turbid emulsion of the precipitate and was unable to produce its full effect.

The original mixture of human serum and anti-human serum had fixed the greater part but not quite all of the mid-piece fraction of the complement. By re-suspending the washed precipitate it was possible to show that the mid-piece taken up by the precipitate was still in an active state and able to take its part in the lysis of red corpuscles if supplemented by the action of a suitable quantity of end-piece.

This experiment shows that a mixture of antigen and antibody fixes or binds the mid-piece but does not use it up or render it inactive. The precipitate in this experiment also took up a considerable amount of the end-piece. The absorbed end-piece could not be demonstrated in the precipitate.

The main result attained by this experiment was to show that the mechanism of the fixation of complement by a mixture of antigen and antibody is essentially the same as the mechanism of the preparation of the two fractions of the complement by Liefmann's carbon dioxide method. In both cases a precipitate is formed which can be separated, washed and shown to contain the mid-piece fraction of the complement.

It will be noticed that great pains were taken to keep the precipitate cold both during and after its formation. When the experiment was attempted at a higher temperature it was not found possible to re-suspend the precipitate in a satisfactory manner. At any rate it was not possible to demonstrate in it the presence of active mid-piece. When the experiment was carried out at 0° C. a fine emulsion of the precipitate was obtained and partial solution of the precipitate appeared to take place. We have found (Ledingham and Dean, 1912) that the same precautions as to the temperature are necessary in carrying out Liefmann's method. The guinea-pig serum is diluted with nine parts of ice-cold water and the mixture is kept in an ice bath while it is being

TABLE VI. (*Part I.*) *Preliminary Precipitation Experiment.*

	Quantity of normal human serum diluted to 0·5 c.c.	Concentration of the human serum in the mixture of antigen, antibody and complement
1	0·1 c.c.	1 in 50
2	0·05 c.c.	1 in 100
3	0·025 c.c.	1 in 200
4	0·0125 c.c.	1 in 400
5	0·00625 c.c.	1 in 800
6	0·003125 c.c.	1 in 1600
7	0·0015625 c.c.	1 in 3200

TABLE VI. (*Part II.*) *Haemolytic Experiment.*

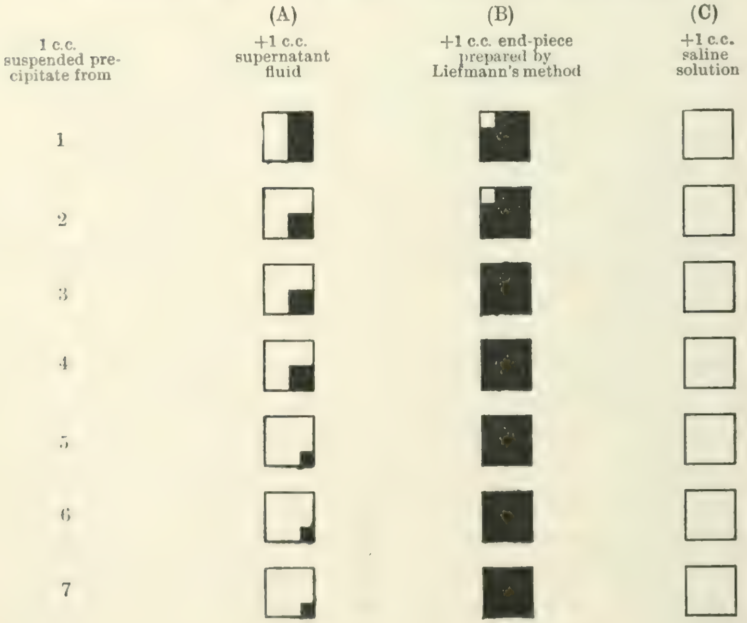
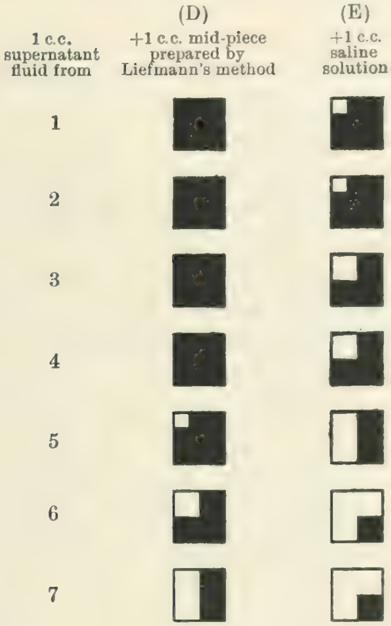


TABLE VI. (*Part III.*)


(D)
(E)
1 c.c. supernatant fluid from
+1 c.c. mid-piece prepared by Liefmann's method
+1 c.c. saline solution
1
2
3
4
5
6
7

saturated with carbon dioxide. The precipitated globulin must be washed with cold distilled water and finally re-suspended in cold saline solution. Unless these precautions are carried out a satisfactory mid-piece preparation is not obtained.

On the relative amounts of mid-piece and end-piece taken up by the various proportions of antigen and antibody.

It will be noticed in the table illustrating the last experiment that the precipitate fixed the greater part but not quite all of the mid-piece. The supernatant fluid on the other hand contained a considerable amount but certainly not all of the end-piece. The experiment recorded in Table VI was undertaken with the object of determining the relative amounts of mid-piece and end-piece taken up by various mixtures of antigen and antibody. The materials employed were normal human serum (antigen), rabbit *v.* man serum (antibody), fresh guinea-pig serum, mid-piece and end-piece fractions prepared by Liefmann's method.

A series of quantities of the human serum from 0·1 c.c. to 0·0015625 c.c. were measured out into seven tubes. The human serum was diluted in every case to 0·5 c.c. The tubes were then placed in the cold room at 0° C. The necessary quantities of antiserum, of complement and of saline were at the same time put in the cold room. At the end of one hour 4 c.c. of a 1 in 8 dilution of the guinea-pig serum was added to each tube and 0·5 c.c. of the undiluted antiserum. The concentration of the antiserum and of the complement was in each tube 1 in 10. The concentration of the antigen in the various tubes is shown in the table.

The antigen, complement and antiserum were mixed in the cold room and then left at 0° C. for three hours. The method was precisely the same as that used in the last experiment (Table VI). The precipitation from each tube was separated from the supernatant fluid, washed, and re-suspended in 5 c.c. of cold saline. A mixture of equal parts of a 1 in 20 suspension of sheep corpuscles with a 1 in 200 dilution of haemolysin was prepared and 1 c.c. of this mixture was added to 1 c.c. of the suspension of precipitate from each of the seven tubes. Three such mixtures were prepared from the precipitate of each of the seven tubes of the original experiment.

At the same time 14 tubes were prepared containing 1 c.c. of the blood and haemolysin mixture and 1 c.c. of the ordinary mid-piece preparation. All these tubes were left for one hour at 0° C. The tubes were then removed from the cold room.

The tubes containing suspended precipitate received in each case either 1 c.c. of the corresponding supernatant fluid, 1 c.c. of end-piece preparation, or 1 c.c. of saline solution. The tubes containing the mid-piece preparation received either 1 c.c. of a supernatant fluid or 1 c.c. of saline solution. All the tubes were then incubated for two hours at 37 ° C. when the results were read and recorded.

It should be stated that the mid-piece and end-piece preparations were carefully tested and shown to be pure.

Column E shows the amount of complement remaining free in the supernatant fluid. The amount of complement fixed increased as the relative concentration of human serum (antigen) in the original mixture was diminished. In no case was all the complement fixed ; in no case was all the mid-piece fixed. The original mixtures, it will be seen, contained a large quantity of complement (0·5 c.c. guinea-pig serum).

Column B shows that the mid-piece had been taken up by the various precipitates and that it remained in an active condition. By comparing the results in columns B and E, it is seen that the amount of mid-piece taken up by the precipitate increased as the concentration of the antigen in the original mixture diminished.

Column D shows that end-piece was fixed, at any rate by the mixtures in Tubes 5, 6 and 7.

The experiment shows that a precipitate produced by the interaction of antigen and antiserum binds both mid-piece and end-piece. The mid-piece is far more readily bound than the end-piece but the experiment shows that traces of mid-piece may remain free after a considerable amount of end-piece has been fixed. After the mid-piece has been fixed by a precipitate it can be still demonstrated in an active condition in the suspension of the precipitate.

It was not found possible to demonstrate the presence of active end-piece in the precipitate and it must be assumed that the end-piece is rendered inactive when it is fixed.

Summary and Conclusions.

(1) If a series of dilutions of an antiserum are prepared, a dilution can be selected which when mixed with an appropriate quantity of the homologous antigen forms no precipitate but nevertheless binds complement. If complement is added to such a mixture of antigen and antiserum a precipitate appears after an interval of six to twenty-four hours.

(2) By using an appropriate mixture of antigen and antiserum the amount of the precipitate can be increased, within certain limits, by increasing the amount of guinea-pig serum (complement) present in the mixture.

(3) Similar results can be obtained if an euglobulin solution prepared from guinea-pig serum is substituted for the normal guinea-pig serum.

(4) A suitable mixture of antigen and antiserum precipitates the euglobulin of guinea-pig serum in a manner which may be compared with the precipitation of euglobulin by carbon dioxide.

(5) By keeping a precipitating mixture of antigen, antiserum and complement at a temperature of 0° C. it is possible to demonstrate that the resulting precipitate contains the mid-piece fraction of the complement in an active state.

The mid-piece fraction is not used up when it is bound by a mixture of serum with its homologous antiserum.

(6) The precipitate which results from the interaction of antigen and antiserum fixes both fractions of the complement. The mid-piece is fixed much more readily than the end-piece but it is possible to demonstrate a stage when a considerable quantity of end-piece has been bound while a small quantity of mid-piece still remains free.

(7) The results obtained in these experiments show that the fixation of the fractions of the complement by a mixture of antigen and antiserum is essentially similar to the fixation produced by suspensions of barium sulphate and similar complement fixing substances. These experiments confirm the recent work of Gengou on this subject.

(8) The particles of a precipitate probably adsorb the euglobulin of the guinea-pig serum and this adsorption of euglobulin is an essential part of the mechanism of complement fixation.

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INFANT MORTALITY
AND ITS
ADMINISTRATIVE CONTROL.

BY

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INFANT MORTALITY AND ITS ADMINISTRATIVE CONTROL.[1]

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THE death of a child appeals in a peculiar way to the emotional side of human nature. Reason may satisfy us that the loss of a young man in the spring-time of his powers or of an adult during the period of full mental and physical vigour is really a greater tragedy, but we seem all of us, at least in the abstract, more touched by the pathos of death at the beginning of life. Most recall with tenderness the lines of Vergil:—

> "Continuo auditae voces vagitus et ingens
> Infantumque animae flentes in limine primo,
> Quos dulcis vitae exsortis et ab ubere raptos
> Abstulit atra dies et funere mersit acerbo."

There was a time when a high rate of infant mortality seemed part of the settled order of the universe; a view expressed with his usual precision by T. R. Malthus, over a hundred years ago.

> "And yet when we contemplate the insufficiency of the price of labour to maintain a large family, and the amount of mortality which arises directly and indirectly from poverty; and add to this the crowds of children which are cut off prematurely in our great towns, our manufactories, and our workhouses, we shall be compelled to acknowledge that if the number born annually were not greatly thinned by this premature mortality, the funds for the maintenance of labour must increase with much greater rapidity than they have ever done hitherto in this country in order to find work and food for the additional numbers that would then grow up to manhood."[2]

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[1] A lecture delivered at University College, London, on February 2[illegible]th, 1912.

[2] T. R. Malthus, "An Essay on the Principle of Population," 5th Edition Vol. II., p. 49.

According to Malthus, 50 per cent. of all persons born in London died under the age of three years,[1] while the average mortality for the first year of life was, in Sweden, 25 per cent.

Period.	1730-49	1750-69	1770-89	1790-1809	1810-29
Percentage Deaths under 5 years.	74·5	63·0	51·5	41·3	31·8

As will be gathered from Table I., in our own times the rate of infant mortality is lower, but still very heavy in the most civilised countries. In the course of the last century public opinion respecting the subject of infant mortality has changed, under the influence of a variety of circumstances. In the first place the emotional aspect of child mortality has certainly not become less prominent; again, the diminution in effective fertility which has been noticed in greater or less degree almost universally has given rise in some minds to anxiety regarding the future of civilisation, an anxiety which has led to inquiries as to whether the effects of a falling birth rate might not be neutralised by a reduction of the infant death rate. Lastly, the triumphs of hygiene over many zymotic and contagious diseases, together with improvements in the whole art of medicine and surgery, have seemed to open up prospects of a successful onslaught upon infant mortality. At the present time, the opinion current in influential quarters might, I think, be summarised as follows:—

A certain number of deaths occurring in the first year of life are due to causes entirely beyond human control. Some children born with grave developmental anomalies of the circulatory or nervous systems are examples; some cases of premature birth are also instances. These cases, however, although absolutely numerous, form but an insignificant proportion of the whole number of infant deaths. The bulk of infant deaths are the result of bad feeding, bad housing, insufficient and unskilled attention, an unhygienic environment in the widest sense of the word.

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[1] For earlier times we have: (see "*Vital Statistics,*" by W. Farr, London, 1885, p. 195).

The removal of these immediately destructive conditions is within the sphere of an enlightened system of public administration, and we may hope, with a sufficient expenditure of money, brains, and energy, enormously to reduce the present rate of infant mortality. In one sentence, a low or a high rate of infant mortality is *mainly* a matter of good or bad public health administration, actual or possible.

That I have not drawn an imaginary picture of opinions advocated by leading members of my profession is sufficiently demonstrated by the following quotation from a work, addressed to the general public, by the well-known physiologist, Professor Benjamin Moore, of Liverpool:—

> "One person out of every five persons born dies before the fifth birthday is reached, and under proper conditions of sanitation at least 80 per cent. of these deaths could be avoided. Latin names figure in the returns of the Registrar-General as the cause of death, but in plain English the children die from dirt, ignorance, carelessness, and starvation. Also, sad to relate, there has not been the slightest improvement in this respect within the past 40 years. Neither is it possible to hug the fond delusion that these are weak, enfeebled children, to preserve whom would be detrimental to the race. These children who die are born healthy, and placed in similar circumstances any other children would succumb just as fast as these. The diseases of infancy fall upon the fit and the unfit; and epidemic diarrhœa or bronchitis caused by neglect after measles, kill the fit just as much as the unfit."[1]

I am not acquainted with the evidence upon which Professor Moore bases his estimate of 80 per cent. of deaths under five years of age due to preventible causes, but, in any case, I am only concerned to show that the view sketched above is held by educated medical men.[2]

Now, we should ask ourselves one question with regard to this doctrine, viz., is the evidence upon which it is based complete? An answer to this question would in no way commit us to an opinion respecting the desirability of the measures advocated by social reformers with a view to diminishing the rate of infant mortality, but would help us to form a judgment as to the amount of success they may be expected to achieve under present conditions of society.

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[1] Prof. B. Moore, "The Dawn of the Health Age," London, 1911, p. 15.

[2] "Some of the principal causes [*i.e.*, of infant mortality] are improper and insufficient food, bad management, use of opiates, neglect, early marriages, and debility of mothers; but whatever may be the special agencies at work which are so prejudicial to infant life, it must be borne in mind that a high death rate is in a great measure also due to bad sanitary arrangements." (Farr, *op. cit.*, p. 192.)

The object of this paper is to direct the reader's thoughts to some of the difficulties which arise in interpreting the evidence at present relied upon by medical inquirers. It is not within my power to remove or lessen these difficulties, but time spent in considering them will not be wasted. Paley, I think, remarked that it is often more troublesome to convince people that a difficulty exists than to show them how to remove it. Perhaps, if I can show that there really is a problem to be solved, some reader may succeed in discovering its solution.

Common sense indicates two great sets of phenomena which are of importance in gauging the chance a child will have of living through one year of existence. The first set comprises the events which precede its birth; the second consists of the post-natal conditions to which it must be exposed. Let us briefly notice these in order:—

(1) To begin with, we have the nexus of cause-groups summarised in common speech by such terms as innate characters, potential characters. We have also the effects which may indirectly be exerted upon the unborn child by the exposure of its mother to an unfavourable environment. For instance, if the mother be underfed or overworked, or both, the circumstances might react unfavourably upon the embryo. We may include here cases of germinal or placental infection, such as syphilis or congenital tubercular disease.

Passing now to (2), post-natal conditions, we have, as before, in the first place, the continued influence of innate factors, bone of the child's bone and flesh of its flesh, we have also the indirect reaction of the maternal characteristics, made manifest perhaps in an inability to suckle the child owing to anatomical or physiological shortcomings. But in addition to these we have the direct action of the environment upon the child itself, a factor which now becomes of striking and, upon superficial examination, overwhelming importance. A new-born child seems, indeed, largely at the mercy of its environment. A physiological machine requiring for the satisfactory performance of its functions a set of conditions varying within but narrow limits, its powers of self-adjustment are of the slightest; thus it has yet to acquire the status of a homoiothermal animal, it needs a highly specialised food, and its means of self-defence are purely vocal. We see that the environmental factors of prime importance may themselves be ranged in two groups. On the one hand, we have to consider the kind of food provided, whether natural or artificial, if the latter, whether it be in quality and quantity approximating to that devised by nature; on the other hand, we have the environment in a more popular sense, including the kind of clothing and shelter as well as the quantity and quality of personal attention afforded by mother or nurse.

Very little reflection is sufficient to show that these environmental factors are themselves the resultants of a chain of causes difficult to isolate. Thus, consider the method of feeding. If the mother does not suckle her child, it may be because she goes out to work and has no time for nursing. If the action be solely conditioned by economic pressure, this cause might be removed. The solution is theoretically simple; let the State pay the family the sum which would have been earned by the mother had she been at work, on condition that she suckles the infant. But this presupposes that the type of the woman that goes out to work is in no way differentiated from that of her home-keeping sister. Perhaps the supposition is well founded, perhaps it is not. A similar ambiguity will be felt when we analyse any other environmental influence.

I have not, of course, given anything like a complete catalogue of the influences which are of importance to infant life. Still, if one considers even those I have named, it is sufficiently obvious that a complete study of the problem is a task of immense magnitude. For the present we must needs confine ourselves to a much humbler inquiry, and we must be critical of those who claim to have solved the whole problem of infant mortality. Let us, then, merely attempt to ascertain the relative importance of those factors which have at different times been brought into prominence by medical and sociological writers. Of such factors, three have usually been accorded special attention, viz., the birth rate, the prevalence of poverty, the prevalence of artificial feeding. A few words must be devoted to each of these :—

The Birth Rate.—This is probably the factor which attracted notice first and its relation to the infant death rate, in the apprehension of Malthus and his immediate followers, may be inferred from the quotation I have already made. Their inference was that the population presses closely upon the means of subsistence, and that consequently the birth and death rates were bound together. It has therefore resulted that when a school of political thought arose which instilled a belief that, even in densely populated countries, the means of subsistence are more than adequate to sustain an enormously greater population than the existing one, the old train of ideas no longer went unchallenged. Even if we assume, however, that the Malthusian doctrine is economically and biologically false, it does not follow that the connection between the birth and death rates is accidental or non-existent. Suppose we sorted out our population into two mutually exclusive classes, one comprising the tuberculous, the criminal, the mentally ill-balanced, and in general all those families some members of which had exhibited a physical or mental stigma; the other class being formed of the rest of the population. Next let us suppose that the characters which distinguish the former group are the outward visible signs of some inward peculiarity, innate and heritable. Also let us suppose that the fertility of the first class is greater than that of the second. What consequences may be expected to ensue? If our tuberculous and insane stocks are so in virtue of an innately low resisting power towards the harmful elements of a common environment, we shall hardly be surprised if their offspring are in turn less fit to combat the ordinary dangers to which infants are exposed than are the children of normal persons; in other words, there would appear to be some correlation between fertility and infant mortality. It will be noted that the assumptions of a differential birth rate and of an heritable type, physiologically inferior to the normal, are essential to this argument. It is unnecessary to observe that numerous publications by the staff of the Galton Laboratory have established a formidable case in support of both propositions. It is necessary to remark that the genesis of the higher birth rate among the less resistant stocks—assuming it to exist—is not a point the discussion of which is germane to my argument. I am only anxious to show that a correlation between the birth rate and the infant death rate *might* arise independently of the economic factor to the operation of which it is usually referred. I do not even go so far as to suggest that this is the real explanation, but merely plead for its being borne in mind when we come to discuss the whole problem.

The Poverty Rate.—The possible influence of bad economic conditions upon the fate of infants, I think I may say the *certain* influence of such conditions, hardly needs illustration. The difficulty in connection with this topic is to obtain a satisfactory measure of what we term poverty, a measure which will be appropriate for statistical use. This, however, is a subject which has attracted the notice of too many writers for it to be permissible to enlarge upon the difficulties here.

The Methods of Infant Feeding.—The evil possibilities of the feeding-bottle, the dangers of septic contamination and the relation between such contamination and the development of summer diarrhœa have often been noted. In addition, we should remark that the whole trend of modern physiological chemistry leads one to emphasize the specificity of animal secretions. Human milk can be prepared nowhere but in the human mammary gland, no chemical ingenuity will suffice to convert the milk of any other species into something "just as good." Here, again, in a factor ostensibly environmental we detect the possible intervention of constitutional causes. As Prinzing remarks: "The causes are either disinclination on the part of the mother, social conditions, or a defective functioning of the mammary glands."[1] It is obvious that physical characters, possibly heritable, may be here of great moment. This brief analysis of the popular explanations of a high rate of infant mortality has sufficed to show that not one of the alleged causes can be regarded as exclusively environmental, and that, were we able to prove that any one of them takes definite precedence of the others as an effective cause, it would not be possible to assert that infant mortality is necessarily within the field of administrative control. At the same time, contenting ourselves with a somewhat superficial view, it might be said that the effects of public health administration are likely to be most marked in connection with the dangers of infant feeding, less plain in connection with poverty, and least of all with respect to the birth rate. The administrator can do a good deal to reform the milk supply, something towards improving the general conditions of life, but, even then he will hardly succeed in raising or lowering the birth rate.

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[1] Prinzing, *Handbuch d. Medizin. Statistik*, p. 202-3.

It, therefore, would seem to follow that were we able to show that the rate of infant mortality is most highly correlated with the practice of bottle-feeding as at present carried out, less closely associated with the rate of poverty and least with the birth rate, we should have grounds for hoping that sanitary and administrative reform might ultimately reduce the infant death rate to some seven per cent. suggested by Westergaard as about the possible minimum.[1] Since the general average is considerably more than 10 per cent., this would mean a great saving of life. How, then, are we to measure the relative importance of these factors?

Quite recently the Local Government Board has issued a most valuable report on the subject of infant mortality,[2] mainly the work of Dr. Arthur Newsholme, the Board's principal medical officer, whose eminence, both as a vital statistician and hygienist, guarantees the care with which the work has been done. I am not concerned with the general aspects of this report, which contains much worthy of close attention, but I should like to quote a few sentences bearing upon the problem which immediately concerns us. Dr. Newsholme measured the correlation between the corrected birth rate and the infant death rate for 46 registration counties and found it to be ·36; commenting upon this, he remarks: "Large families evidently do not necessarily imply a tendency to high infant mortality. They should *ceteris paribus,* except in circumstances of extreme poverty, have an opposite effect to a slight extent. The connection often observed between a high birth rate and a high rate of infant mortality probably is due in great part to the fact that large families are common among the poorest classes, and these classes are specially exposed to the degrading influences producing excessive infant mortality."[1]

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[1] Westergaard, *Die Lehre v. d. Mortalität und Morbidität*, 1901, p. 403.

[2] Supplement to the Report of the Board's Medical Officer, 1910, Cd. 5263.

Should this conclusion be just, it would follow that the correlation between the birth rate and the rate of infant mortality ought to be much reduced if the factor of poverty could, by some means, be kept constant. Notwithstanding the eternal difficulty of finding a measure of poverty, it would seem that some approximation to the end in view might be hoped for through the use of the calculus of correlations. Apparently, however, no suitable data exist in this country, for neither poverty nor the prevalence of artificial feeding is brought into relation with infant mortality by means of the method of correlation in the Local Government Board report. A difficulty which confronts anyone attempting to solve the problem upon the basis of English official records are the size and heterogeneity of the conventional subdivisions, the registration counties. When it is a question of the general features of infant mortality all over the land (as was the case in the L.G.B. report), the objection may be of little moment. If, however, we set out to study the relative significance of the three factors enumerated, the introduction of such modifying conditions as greater or less urbanisation, differential employment in various industries and even climatic conditions increase the difficulties of our task.

For these reasons, it appeared necessary to find a set of communities not very different one from another in regard to the type of inhabitants, their occupations, and general conditions of life. With such material, a first study might begin and might suggest lines along which the general problem could be approached. To anyone familiar with the present state of medical statistics, it is unnecessary to say that the conditions I have postulated are not fulfilled by any readily accessible collection of records. I did, however, obtain some material which approximated more closely to my requirements than any other figures I had seen. These data were particulars of the rural districts of the kingdom of Bavaria, published by Drs. Groth and Hahn in a recent paper.[1] In this work we have (1) the Birth Rate; (2) the Infant Death Rate; (3) the Poverty Rate, as measured by the proportion of persons in receipt of public assistance; (4) the Prevalence of Artificial Feeding, as indicated by the proportion of the children brought to the public vaccinators which had been bottle fed (in a large number of districts). Naturally these data are not ideal. The proportions of artificially fed children brought to the vaccinating stations only measure the prevalence of bottle feeding in a certain class of the population and even this datum is wanting in many districts. The measure of poverty, although superior to similar returns of pauperism in this country owing to the probably greater uniformity of the system in Germany, can easily be criticised. There are various other criticisms which will be found discussed elsewhere. Nevertheless, making the most liberal deductions from the face value of the data, I think something of interest can be extracted from them with regard to our present topic, and I shall describe certain results obtained on analysis by my assistant, Mr. J. W. Brown, and myself.

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[1] *Op. cit.*, p. 49

To begin with, we determined the correlation between the four variables taken in pairs, and then between each variable and the absolute population; lastly, the average correlations for a constant population were ascertained. For instance, the correlation between the poverty rate, as measured by Groth and Hahn, and the infant death rate was found; then the correlation between the population and the poverty rate of each district and between the former and the infant death rate. Finally we calculated the partial correlation between the infant death rate

[1] Groth and Hahn—*Die Säuglingsverhältnisse in Bayern,* München, 1910 (Lindauer) (*Sonderabd. a.d. Zeit. d. K. Bayer. Stat. Landesamt,* Jahrg., 1910).

[2] Greenwood and Brown, "An Examination of Some Factors Influencing the Rate of Infant Mortality," *Journ. Hyg.*, 1912, Vol. XII., pp. 5-45.

and the poverty rate for a constant value of the population. This method, to some extent, corrects the error arising from the fact that we are correlating ratios and *pro tanto,* introducing some amount of possibly spurious correlation. Table II. contains the results deduced. The birth rate was based upon the total number of women aged 16-50 in each district, an imperfect method but somewhat superior to the ordinary crude birth rate. From these results, it appears that the rates of artificial feeding and birth are highly correlated with the infant death rate, which is also markedly but less closely associated with the poverty rate. We endeavoured to check these results against the values of similar coefficients deduced from other data, as will be seen in Tables III. and IV. Leaving on one side the coefficients based upon English registration countries and remembering the difficulties introduced by varying methods of measuring poverty and calculating birth rates, it is not clear that the comparison should lead us to discredit the coefficients obtained from Groth and Hahn's material.

We now come to the most interesting part of the work, viz., the relative importance of the different factors, and are at once brought face to face with a serious difficulty. In order to solve our problem we require the partial correlation between any two variables for constant values of the remaining two.[1] But, as seen in the table, the birth rate and the infant death rate can be correlated in 156 districts, the death rate and the prevalence of artificial feeding in only 97, no returns of artificial feeding being procured from the remaining 59 districts. If we confine ourselves to the districts in which all four measures are recorded, the data shrink to 84 entries.

If we use the total coefficients based upon varying numbers of districts, we disregard the possibility of some selective agency having been at work leading to failure of records for certain variables in certain districts, and there is also some difficulty in ascertaining the probable errors of the partial coefficients. If we confine ourselves to the 84 districts, we suffer the qualms of conscience which assail any statistician who omits data from his series. Evidently the proper course to pursue is to calculate the partial coefficients in *both* ways and see how the results differ. We did this, and the important constants are given in Table V. The differences between the values yielded by the two sets of figures are of some theoretical interest, but I will confine myself to the conclusions which both methods agree in yielding.

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[1] *i.e.,* a measure of the degree to which A and B are associated apart from the indirect association due to a correlation of A with C and of B with C.

They agree in placing poverty last in the scale of relative importance, and in classing the method of feeding and the birth rate as sensibly equal factors of the infant death rate, as measured by the coefficients of partial correlation. If we go a step farther, and attempt to connect changes in the death rate with changes in the other variables by means of a simple equation, then, owing to the fact that the proportion of artificially fed children varies much more from district to district than do either the birth or poverty rates, we find that the birth rate very definitely takes precedence of the method of feeding in order of importance. I do not, however, wish to lay stress upon this latter consideration—which is discussed at length in the memoir by Mr. Brown and myself—since its validity involves certain assumptions which may be questioned; confining ourselves to the coefficients of correlation alone, some reflections necessarily suggest themselves.

In the first place, while the absolute association of poverty, so far as this can be indicated by Groth and Hahn's measure, with the rate of infant mortality is considerable, the importance of the poverty rate as a factor is less than usually supposed. In the second place, there is room for considerable doubt as to whether, in the Bavarian rural districts, the prevalence of artificial feeding is more closely associated with infant mortality than is the birth rate. In other words, these data, so far as they go, hardly bear out the suggestion made in the Local Government Board's report as to the real genesis of the correlation between the birth and infant death rates. In itself, the correlation between the two rates might be looked on as a mere arithmetical curiosity, of no more importance than the number of postage stamps required to pave London Bridge; but if there is really any force in the contention that inferior stocks are the more fertile, we cannot dismiss the results in this way. It is common knowledge that artificial restrictions upon fertility are employed to a considerable extent among the middle classes at the present time. It is fairly certain that this statement would not have been true fifty years ago. Let us suppose that it also holds for many of the efficient members of the working class. The result would be that fertility is now associated with mental and physical inferiority owing to the voluntary adoption of a certain line of conduct on the part of a section of the community. We should accordingly expect to find a difference between the correlations of birth and infant death rates as deduced from modern statistics or material of fifty years ago. This is precisely what Dr. Heron found in the case of the London boroughs. The correlation between the two rates was $+·51 \pm ·10$ for 1901 statistics, $-·30 \pm ·10$ for the data of 1851.[1] It is, *a priori*, improbable that fertility in general would be associated with a lessened power of resistance on the part of the offspring since, were this to be the case, the evolution of a stable type would seem to be difficult (this I take to be the idea prompting the first sentence of the passage quoted from Dr. Newsholme's report). From data, probably little affected by artificial restrictions upon fertility, Beeton, Pearson, and Yule have shown that longevity and fertility are correlated,[2] and there is reason to suppose that longevity is a heritable character.[3] It is not clear that fertility and the rate of mortality are generally associated. In the statistics of upper and middle class families discussed by Ansell in 1874[4], for instance, out of 10,000 born, the following are the deaths in the first year of life in each of Ansell's classes: Clergy, 738; Legal, 797; Medical, 867; General, 843. The mean numbers of children to a marriage (including still-births) in each class are, in the same order, 5·36, 5·32, 4·96, 5·50. It is also clear from the international statistics collected by Prinzing[1] that changes from year to year in birth and infant death rates are by no means uniformly related.

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[1] Heron, *On the Relation of Fertility in Man to Social Status, etc.*, London, 1906, p. 18.

[2] Beeton, Pearson, and Yule, *Proc. Roy. Soc.*, LXXVII., 1901, p. 159.

[3] Beeton and Pearson, *Proc. Roy. Soc.*, LXV., 1899, p. 290. *Biometrika*, I., 1901, p. 50.

[4] C. Ansell, Jun., *Statistics of Families in the Upper and Professional Classes*, London, 1874.

The suggestion I am endeavouring to convey is that the correlation between the infant death and the birth rate is a phenomenon which may well be due to special circumstances, but that it is by no means lightly to be dismissed as a secondary consequence of some environmental factor readily amenable to administrative control. Granted that it is possible to free the poorest classes from those "degrading influences" referred to by Dr. Newsholme, it hardly seems to follow that the connection between a high birth rate and a high infant death rate will be broken.

Let us now pass to the high correlation between the prevalence of artificial feeding in Bavaria and the infant death rate.

Since there is ample room for improvement in the customary methods of hand feeding among the poorer classes, and since some at least of these improvements can be expedited by administrative reforms, the magnitude of the correlation affords us grounds for anticipating a reduction of the rate of infant mortality in the future as a consequence of environmental changes. That this is a justifiable hope, few will dispute, but we must bear in mind certain doubts which a close examination of the facts can hardly fail to produce. Altogether apart from purely statistical considerations, the subject is not free from ambiguities. Let us recur for a moment to the possibility that an incapacity to suckle may be an indication of some constitutional defect. If this be the case, and if further we admit that constitutional peculiarities are heritable, another question must be asked, viz., is the low resisting power of a bottle-fed infant entirely due to the form of its nourishment or may it be possible that inheritance comes into play? If we adopt the view that both factors are involved, how can their respective shares be determined?

There is some, perhaps not much, evidence that an incapacity to suckle is an inherited character. The well-known physiologist, Prof. G. v. Bunge, has identified himself with this view. V. Bunge classified nursing mothers as " Befähigten," or " nicht Befähigten," let us say, efficient or inefficient nurses. The former group comprised the women who had nursed all or the majority of their children for at least nine months, a somewhat stringent qualification. He then distributed among his medical friends schedules with the object of obtaining statistical data. From the data published in his 1904 paper[1] I extracted the information given in Table VI., where a + sign indicates efficiency and a − sign inefficiency.

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[1] Prinzing, *Jahrbuch für Nationalökon. und Statistik,* III. F. Bd. XVII., 1899, p. 577.

For various reasons, it hardly seemed desirable to spend much time in analysing v. Bunge's returns, but they suggest that the subject is one deserving more attention than it has apparently received in this country. Before we can confidently assert that the non-success of hand-fed children is simply an affair of environment, further study would seem to be essential.

We are now in a position to consider the import of the topics here mentioned. First of all, let us be clear as to what we are actually discussing. That feeding with pure milk is less dangerous than using dirty feeding bottles containing adulterated and septic milk, that a child, whatever its ancestry, brought up in a good home is more likely to survive than one entrusted to dirty and poverty-stricken persons, are palpable facts; to doubt about them is not to be sceptical but to be silly. But we have not to deal with elementary truisms of this kind. What we must ask is, whether, in such a world as the present, approximations to good or bad environmental conditions, possible in the existing state of society, are pre-potent factors of the infant death rate. Stated in this way, and I do not see in what other way the problem can fairly be stated, it will appear that our only hope of attaining to a solution is by means of statistical inquiry. The truth of this contention has been effectively admitted by nearly every writer. Almost every argument (leaving out of account platform speeches and other types of ecstatic literature) tending to show that the infant death rate is a function of the environment has been based upon or accompanied by statistical records. An examination of this class of literature would soften the condemnation a reader may be inclined to pronounce upon the inadequate character of the evidence here cited. For me to assert that I have demonstrated the incorrectness of accepted opinions would be absurd presumption. That, however, a belief in the necessity for care in interpreting the evidence before the public is not the whim of an unknown writer will be sufficiently proved by a quotation from the work of a distinguished authority, Dr. Westergaard. After remarking upon the apparent connection between fertility and infant mortality, Westergaard observes:—

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[1] V. Bunge, Virch. Archiv. CLXXV., 1904, p. 185.

> "Here we have displayed a factor of as far-reaching importance as the method of feeding, and any inquiry into infant mortality in order to be based upon firm foundations must pay due regard to both factors, which is unfortunately in most cases impossible. Now, we must not lose sight of the fact that these two factors may themselves perhaps be in part only secondary phenomena. The artificial nourishment may be due to the mother's weak constitution, in which event the higher death rate can partly be explained as an inherited stigma (Belastung). On the other side can be urged that feeding by a wet nurse, which is often due to the same cause, gives, according to Böckh's inquiries, often very favourable results. Social and economic conditions may also intervene as correlative factors. And, with respect to the height of the birth rate, it is tempting to refer the increase of mortality partly to an overtaxing of the maternal organism partly to economic difficulties which often confront the attempt to supply favourable conditions of life for a large family; but we dare not overlook the fact that an opposing hypothesis can be advanced, viz., that it is not the many children which produce the abnormal mortality but conversely the high mortality which calls into being the numerous children in that the loss of children physiologically and psychologically renders possible and is associated with a further production of children. It is therefore necessary to burrow deeper in order to separate the individual factors one from the other; this at least stands firm, that where much life is sown still more death is sown, and in particular when the number of children greatly exceeds the average, death claims an abnormal harvest."[1]

This passage from the works of a learned and experienced statistician surely deserves notice. We may ask whether such considerations have always been in the minds of writers upon the subject and whether those who have the heavy and responsible task of educating public opinion might not do well to insist upon the necessity for caution and reserve in interpreting statistical data.

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[1] Westergaard, *Die Lehre von der Mortalität und Morbidität*, Jena, 1901, p. 368.

If one looks at the problem not merely from the standpoint of the specialist who cannot, with the best will in the world, avoid a tendency to over-estimate the importance of conclusions apt to be elicited by processes which he admires and uses and to slight other equally important truths not reducible by his methods, there is still room for much more hesitation than seems fashionable.

What may be termed a collective sense of pity, the will to bring light to them that sit in darkness, to raise those who have been struck down in the battle of life, is a development of the national conscience which few, outside a tiny circle of extremists, would desire to arrest. Even were it true that public efforts to lower the rate of infant mortality by increasing the amount of attention officially devoted to nurslings did not produce all the results claimed for them, it does not follow that they should be diminished. But we must remember that the bulk of persons with whom ultimately the decision rests, those who find the money, are neither very highly educated nor very logically minded. If the public spirited men and women appealing to their fellow citizens on behalf of the children make exaggerated claims with respect to the measures they advocate, they may at first receive more support than would be accorded to modester pretensions. In the long run, however, a nemesis will overtake them. There will be the usual revulsion, the customary recoil from exaggerated credulity to exaggerated scepticism. Before now, useful therapeutic measures have been discredited in consequence of the exaggerated claims made on their behalf in the first flush of enthusiasm.

I firmly believe that all possible measures to improve sanitation, to improve the conditions of slum children, to minimise the chances of spreading infectious disease, the whole programme of the modern medical publicist, should be unflinchingly supported. If we wished to discredit them, the readiest means would be to go up and down the land asserting that by doing this or that, consumption can be "conquered"; that by spending a million here and creating an official there, infant mortality can be reduced by eighty per cent. And so, in the name of sanitary reform itself, I would plead for more criticism of the arguments by which it is supported. At present there is far too much invective and too little discussion in connection with public health matters, signs of the temper which unhappily seem to characterise political controversies. It should be possible for students of science to discuss grave questions affecting the national welfare and to canvass differences of opinion without the use of any expressions which generate much heat and little light. We shall do wisely to live in the spirit of that phrase which Professor Karl Pearson chose as the motto of his famous "Grammar"—*la critique est la vie de la science.*

TABLE I.

Number out of 100,000 born who survive one year.

Country.	No. per 100,000.
Sweden	88,917
France	83,674
Belgium	83,114
England and Wales	82,814
Massachusetts	82,767
Holland	82,681
Italy	82,481
Germany	76,614
Austria	75,028

TABLE II.

Rural Districts of Bavaria.

Variables.	No. of Obs.	Coeff. of Correl.[1]
Infant Mortality and Birth Rate	156	·709 ± ·027
Infant Mortality and Poverty Rate	145	·475 ± ·043
Infant Mortality and Artificial F'd'g Rate	97	·760 ± ·029
Birth Rate and Poverty Rate	142	·420 ± ·047
Birth Rate and Artificial F'd'g Rate	92	·474 ± ·054
Poverty Rate and Artificial F'd'g Rate	87	·379 ± ·062

[DATA COLLECTED BY DRS. GROTH AND HAHN.]

[1] For the purposes of this study, it will be sufficient for the non-statistical reader to regard a coefficient of correlation as an arithmetical constant measuring the extent to which two characters tend to vary concomitantly. The coefficient lies between 0 and ± 1; the nearer the value comes to ± 1, the closer the association (if the sign is positive, the characters increase together; if negative, the increase of one is associated with the decrease of the other). The fraction in the tables preceded by "±" is the "probable error," and but little weight ought to be assigned to any coefficient less, in absolute magnitude, than three times its probable error.

TABLE III.

Poverty and Birth Rates.

Data.	Nature of Birth Rate.	Measure of Poverty.	r.	Computer.
27 London Boroughs	% Married Women 15-54	General Labourers per 1,000	·52 ± ·10	Heron
,, ,,	,, ,,	Pawnbrokers, &c., per 1,000	·62 ± ·08	,,
,, ,,	,, ,,	Prop. living more than 2 in a Room	·70 ± ·07	,,
,, ,,	,, ,,	Prop. of Children aged 10-14 employed	·66 ± ·07	,,
142 Rural Dis. Bavaria.	% All Women 16-50	% Publicly assisted persons.	·44 ± ·05	G. & B.
22 Districts of Hamburg.	% Married Women 15-45	% Dwellings rented less than 300 marks	·88 ± ·03	,,
26 Districts of Leipzig.	% All Women 15-40	% Dwellings rented less than 250 marks	·83 ± ·04	,,
26 Districts of Leipzig.	% All Women 15-40	% Dwellings less than 2 Heated Rooms	·91 ± ·02	,,

TABLE IV.

Correlation of Birth—and Infant Death—Rate.

Source.	Computer.	Correlation.	Birth Rate.
27 London Boroughs	Heron	·50 ± ·10	Married Women 15.54
38 Cities Mass.	G. & B.	·42 ± ·09	Crude
704 Rural Districts German	G. & B.	·58 ± ·02	Crude
156 Rural Districts Bavarian	G. & B.	·68 ± ·03	All Women 16.50
143 Cities Saxon	G. & B.	·42 ± ·05	Crude
44 Reg. Counties English	Newsholme	·36 ± ·09	Corrected
44 Reg. Counties English	G. & B.	·64 ± ·06	All Women 15.45

TABLE V.

Partial Correlations of the Rate of Infant Mortality with other Variables.

Characters.	Correlation.	Coefficients.
	A Varied Totals.	B Select 84.
Infant Mortality and Birth Rate	·57 ± ·05	·70 ± ·04
Infant Mortality and Artificial F'd'g Rate	·66 ± ·04	·62 ± ·05
Infant Mortality and Poverty Rate	·17 ± ·07	·32 ± ·07

[RURAL DISTRICTS OF BAVARIA. GROTH AND HAHN.]

TABLE VI.

[*G. v. Bunge, Virch. Arch. CLXXV.* 185.]

MOTHERS.

		+	—	
DAUGHTERS.	+	422	1	423
	1	281	435	716
		703	436	1139

ON METHODS OF RESEARCH AVAILABLE IN THE STUDY OF MEDICAL PROBLEMS

WITH SPECIAL REFERENCE TO SIR ALMROTH WRIGHT'S RECENT UTTERANCES

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ON METHODS OF RESEARCH AVAILABLE IN THE STUDY OF MEDICAL PROBLEMS,

Introduction.

MANKIND are pretty well agreed that attempts on the part of those unversed in the technic of any branch of scientific inquiry to lay down precise rules for the guidance of actual workers, and to formulate laws descriptive of the processes by which truth is attainable, do not sensibly improve the conditions under which scientific research is carried out. On the other hand, when the methodologist is himself an investigator of high standing, whose name is associated with genuine achievements in the domain of science, it is rightly held that his conception of the means best calculated to attain definite scientific ends at least deserves the serious attention of other workers. For these reasons the utterances of Sir Almroth Wright merit careful examination. It will hardly be disputed that Sir Almroth Wright is a leader in a branch of scientific medicine highly esteemed by some of the best contemporary minds, and it is perhaps hardly less doubtful that the present state of medical science is not altogether satisfactory. The latter circumstance may be attributable to a failure to grasp both the ends capable of being reached and the means to be preferred. If Sir Almroth Wright has thrown light upon these matters, no insignificant item has been added to the world's debt of gratitude to him.

Sir Almroth Wright has recently enunciated various methodological conclusions of far-reaching importance, and I propose to attempt an examination of their scope and validity. It will, perhaps, appear an act of presumption on my part to enter the lists with so redoubtable an adversary, but some justification is afforded by the following circumstances. Sir Almroth Wright has satisfied himself that the application of statistical or biometric processes to medical problems cannot yield trustworthy results. The number of biometrically trained medical men is still small, and, so far as I am aware, I am the only one in this country holding a post expressly created to further the application of biometric methods in medicine and pathology. I seem, therefore, honourably obliged either to acknowledge the justice of Sir Almroth Wright's conclusions or publicly to state the reasons which lead me to think them unsound, and to submit for criticism the principles I hold to be just.

One further preliminary remark may be desirable. I believe that honest controversy is of value to science. The contempt in which the former is increasingly held is due to the ebullitions of temper and the direct or veiled attributions of unworthy motives which mar so many discussions.

The present topic is highly controversial; I have endeavoured to bear in mind the words of Cromwell: "I beseech you, in the bowels of Christ, think it possible you may be mistaken." If, however, in the heat of conflict or the vanity of authorship I have used any expression unseemly in one discussing a vast and complex subject with an older and abler man, I beg that the offence may be attributed to carelessness, not to malice.

I. *Sir Almroth Wright and the Opsonic Index.*

Sir Almroth Wright's final pronouncement on the rightful place of a statistician is to be found in his "Report on the Pharmaco-therapy of Pneumococcus Infections, and on the Methods by which such Therapeutic Problems ought to be Investigated,"[1] but before we consider this report in detail it is necessary to form a clear idea as to what its author really takes to be the statistical method and as to his attitude towards statisticians in a concrete case. Very fortunately, an example of the latter is to hand, and, indeed, Sir Almroth Wright's recent generalisations were, if not inspired, at least stimulated, by a contemplation of this particular case. I therefore propose to examine it in some detail.

Some years ago, in 1907–08, a discussion arose (not by any means the first which had taken place) respecting the value of determinations of the tuberculo-opsonic index. The exciting cause was a paper by Miss M. P. Fitzgerald, Mr. R. I. Whiteman, and Mr. T. S. P. Strangeways[2] in which the value of such determinations was seriously questioned. Being at that time in touch with enthusiastic advocates of Sir Almroth Wright's methods, I naturally heard a good deal of talk about the matter, and the impression I derived was that no one had very definite ideas as to how wide the limits of normal variation might be when the test was carried out by competent technicians. Some thought that all indices beyond the limits 1·1 and 0·9 might be regarded as strong, if not convincing, evidence of abnormality; others made even higher claims for the accuracy of the process. Since the most casual inspection of actual films or even of the published writings of observers showed that the phagocytic content varied from cell to cell; since, further, divergences of opinion between authors, each of whom had at least *primâ facie* claims to speak with "authority," were considerable, it seemed that some degree of accuracy should be imported into the discussion by an analysis of phagocytic counts. Had I been one of the purely mythical "statisticians" who bulk so largely in Sir Almroth Wright's more speculative pages, I suppose I should have employed a laboratory servant to count cells on films prepared by myself and have speedily amassed data which would have been, in point of numbers, everything that could be desired. Actually, I applied to Sir Almroth Wright himself, explained the object of my inquiry, and asked him to supply data which would be above the suspicions freely bandied about respecting Strangeways's material. Sir Almroth Wright kindly acceded to my request and sent me records of cells prepared and counted by his assistant Mr. Alexander Fleming. Mr. Strangeways also placed his material at my disposal, and I selected therefrom certain records which corresponded in point of numbers counted with those of Mr. Fleming. I did not realise at the time, but have since ascertained, and now explicitly state (as we are engaged in a scientific discussion, not a comic opera, there can be no reason for secrecy), that these slides also were prepared in Sir Almroth Wright's laboratory, although they were enumerated by Mr. Strangeways. The material therefore at my disposal consisted *entirely* of slides prepared in Sir Almroth Wright's laboratory, and partly of slides both prepared and counted in that laboratory. Dr. Douglas White and I then analysed these data by biometric methods, this work forming the subject of our first memoir in *Biometrika.*[3] The results so far as they affect the present issue were as follows. 1. Phagocytic counts have pronouncedly asymmetrical distributions. 2. The means of samples of 25 are also asymmetrical in distribution and, in samples of this order, deviations outside the limits 1·2 and 0·8 may frequently occur in normal material. 3. There was no reason to suppose that Strangeways's method of enumeration led to worse results than that of Fleming. The inferences we drew from these results were firstly that the variability of the opsonic index in normal sera might well be greater than Sir Almroth Wright's adherents had realised; secondly, that such variations as Strangeways and his collaborators had found could not fairly be held to flow from their unskilful methods of counting.

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[1] THE LANCET, Dec. 14th and 21st, 1912.

[2] Bulletin of the Committee for the Study of Special Diseases, vol. i., 1907, No. 8 (Cambridge).

It is worth noting that quite independently Major W. F. Harvey, I.M.S., and Captain A. G. McKendrick, I.M.S.,[4] who also employed biometric methods, concluded that the tuberculo-opsonic index might be of value *when the index fell beyond the limits 1·2 and 0·8.*[5] It is proper, however, to add that these authors expressed a more favourable opinion of the opsonic method in general than in the case of the tuberculo-opsonic index in particular. I may as well state here that I am not in a position to offer any opinion whatever as to the accuracy of the opsonic index in connexion with any organism but the tubercle bacillus. Both Dr. White and I have been frequently assured that other organisms are far more satisfactory material for inquiry, and urged to apply biometric methods thereto. Three considerations have deterred me from following this advice. The first is that the value of the opsonic test has been, and still is, rated very high by Sir Almroth Wright in the diagnosis of tubercular infections and as a control in their treatment; the second is a memory of certain adventures of Isaac detailed in the twenty-sixth chapter of Genesis; the third a disinclination to spend an inglorious old age in the reduction of endless phagocytic counts.

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[3] Biometrika, vol. vi., 1909, p. 366.

[4] Biometrika, vol. vii., 1910, p. 64.

Returning now to the conditions created in our minds by the result of our first analysis, it seemed plain that further work required to be done before we could hope to form an exact idea as to how variable the opsonic index really was, since we required a much larger number of cells than had so far been dealt with. To fulfil our conditions we required an enumeration of some 20,000 cells, and it did not seem reasonable to expect other workers not interested in our methods and sceptical as to their value to undertake so laborious a task. My collaborator, Dr. White, accordingly did the work himself. Dr. White had had 18 months' previous experience in the inoculation department of the London Hospital; in other words, at least as complete a training in the special technic as many gentlemen whose opsonic estimations have been received with respect. We might therefore have urged that the onus of proof lay upon our critics, and that they should demonstrate, not assume, the enormous "functional errors" vitiating White's counts. We were, however, fully alive to the fact that had the count been specially made by an archangel and yet revealed on analysis sensible departures from the model furnished by the Fleming counts, it would have been instantly rejected as worthless by Sir Almroth Wright and his followers. We accordingly made a careful comparison of the form of distribution revealed by our count and the types conformed to by the great exemplar from St. Mary's (incidentally we postponed the publication of our results for some months until we had specially considered the effect of one departure from the previous technic). Such differences as we noticed seemed of little interest to any but biometricians ; in the particulars essential to the objects of the inquiry there was no divergence not attributable to errors of random sampling or the thickness of the bacillary emulsion employed. Fortified by the results of this examination we seemed to be justified in attempting to answer the vital question, What are the limits of variation within which the opsonic index will be likely (the grades of likelihood being expressed quantitatively) to fall when a serum is tested against itself, the index being based on counts of 25, 50, or 100 cells? That is to say, With what frequency may specified deviations from the theoretical value, unity, be expected to occur? This is plainly the simplest test that can be employed, since the possible variations of opsonin in *different* normal sera, which would in practice introduce a further source of error, are excluded.

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[5] Their words are: "The tuberculo-opsonic index is capable of giving in routine practice definite indications of the positive or negative opsonic activity of serum *for values which range outside 0·8 and 1·2.*" (The italics are mine.)

Naturally having given the distributions of the original quantities used in forming the ratio called an opsonic index, the question is theoretically capable of direct algebraical solution. This was the method by which we first proceeded, but the formula deduced by us was not, for theoretical reasons, satisfactory, and we fell back upon an empirical process, fully described in our memoir ; we determined the required limits by actual trial. The results obtained were as follows.[6] 1. The odds against a second sample of the same material giving an opsonic index in terms of the true mean outside the limits 1·2 and 0·8 are in the case of samples of 25 about 3 to 1, in the case of 50's about 6 to 1, and for 100's about 15 to 1. 2. When the index is estimated on the basis of two small samples and without determining the mean of the normal series from a long count (that is to say, when an index is determined in the ordinary course of routine work) the odds are reduced to 1·5 to 1, 2 to 1, and 4·6 to 1 respectively, for samples of 25, 50, or 100. 3. Even in the case of samples of 100, it is doubtful whether *single* determinations which give values even beyond the limits 1·3 to 0·7 can be regarded as altogether satisfactory evidence of differentiation (the odds against such a deviation occurring as an error of random sampling in normal material are 19·5 to 1, that is, the event is not a very improbable one).

These findings suffered from the weakness of being based upon a small number of trials out of the infinite number of possible trials, but subsequently Pearson[7] deduced a formula, free from the limitations of our own, which allowed the first method—i.e., that of deduction from the constants of the original frequencies—to be adopted. The agreement between his results and our own proved, on the whole, remarkably close. Sir Almroth Wright had up to this point made no public comment upon our findings, but he now, in speaking of those who had criticised the employment of the opsonic index, observed, *inter alia*:—

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[6] Biometrika, vol. vii., 1910, p. 505.

[7] Ibid., p. [illegible]31.

> I suggest that those critics who have put forward figures showing enormous working errors in opsonic estimations may have supplied to the world data with regard to the magnitude of their own functional errors, instead of as self-esteem assured them—data with regard to errors inherent in the opsonic method.[8]

Professor Karl Pearson, who naturally concluded from this remark (assuming it to be applied to our work amongst that of other critics) that Sir Almroth Wright was not satisfied with our conclusion that the White count conformed sufficiently to the standard of St. Mary's from the point of view of technique, requested me to supply him with data in order to permit of an independent investigation of so important a point. I accordingly forwarded the material to Professor Pearson's laboratory, and an analysis following somewhat different lines from that of White and myself was undertaken. The results of this inquiry were as follows[9]:—

> 1. The same degree of variation in the opsonic index of an individual tested against himself arises when external investigators count slides prepared inside or outside Sir Almroth Wright's laboratory. It seems improbable, therefore, that "enormous working errors" are functional errors due to the defective preparation of the slide.
>
> 2. The same degree of variation in the bacilli per leucocyte arises when the slides are prepared and counted (*a*) inside or (*b*) outside Sir Almroth's laboratory. The population of a slide therefore shows the same high degree of variation under both conditions.
>
> 3. The variation of 20 independent means of 50 from the same individual is of the same order in the case of internal and external workers. The variation of the opsonic index of the same patient tested against himself is as great for the slides tested whether they be of internal or external origin.
>
> 4. There is a difference in the nature of the written records of the counts. The "external" counters worked backwards and forwards across the slide, recording continuously by aid of a mechanical stage. I have no knowledge of how Dr. Fleming's record was entered, but, while it exhibits the same order of variation in the bacilli per leucocyte, the large and small numbers of bacilli appear to be distributed more at random within the groups of 50 in the record.
>
> 5. The results reached are consonant with the "enormous working errors," being for the most part the variations of random sampling; they are not consonant with any wide differentiation resulting from defective technique or from serious individual peculiarities in counting and recording.

It will be seen that these findings amount to an independent confirmation of our principal conclusions.

Perhaps stimulated again by this paper, Sir Almroth, in his recent "Handbook of the Technique of the Teat and Capillary Glass Tube,"[10] honoured White and myself with somewhat more direct notice than formerly. It is true that no bibliographical references are given in the appendix to Chapter IX., entitled "A Consideration of the Contention of the Mathematical Statistician that he has Authority to Pronounce Judgment upon the Number of Leucocytes which Require to be Counted in the Opsonic Film," but, as my name is the only one appearing therein, it is not an unfair presumption that the strictures passed have special application to the papers of White and Greenwood. The language used by Sir Almroth Wright in this section will, I think, be valuable material for those who desire to judge fairly between him and us.

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[8] Wright: Vaccine Therapy, Its Administration, Value, and Limitations, Proceedings of the Royal Society of Medicine, vol. iii., 1910, p. 29 of reprint.

[9] Biometrika, vol. viii., p. 203.

[10] London, 1912, Constable and Co.

On p. 164 of his book, after drawing attention to the fact that the word "significant" is employed by different people in different senses, and that some readers might infer, when these terms are used, that the user has some scientific resources at his disposal which allow him to state whether certain differences are or are not the effects of random sampling, Sir Almroth Wright says:—

> It cannot be too clearly understood that the mathematical statistician has no such secret wells of wisdom to draw from, and that his science does not justify his going one step beyond the purely numerical statement that—as computed by him from the data he has selected as suitable for his purposes—the probabilities in favour of a particular difference being or not being due to the operation of chance are such and such.
>
> There need, therefore, be no hesitation in saying that when the mathematical statistician makes free with the terms *significant* and *non-significant*, he is simply taking upon himself a function to which he can lay no claim in his capacity as a mathematician.

It is well to remember that the majority, the immense majority, of Sir Almroth Wright's readers would know precisely as much about the statisticians' work as he chose to tell them; indeed, he does not, by citing the papers criticised, even put his readers in the way of studying the stigmatised works at first hand. The question is, therefore, what inference an ordinarily intelligent reader would draw from the words quoted. I think the inference must be that the statisticians have used a common word in a specialised sense, and have consciously or unconsciously misled the ordinary reader. It would be inferred that if the statisticians had defined their terms Sir Almroth Wright would have said as much. Some students of Sir Almroth Wright's book may therefore be surprised to learn that in one of the papers of White and Greenwood the following passage occurs:—

> If we adopt about a ten to one chance as the limiting value for evidence of differentiation, then the limits for single determinations of the index are roughly ...... Everyone has his own standard of accuracy or reliability; all we mean is that unless a given index is beyond these limits, the chances are less than ten to one against its being drawn from a normal population.[11]

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[11] Biometrika, vol. vii., p. 520.

Again, in a preliminary note intended for the lay reader, I remarked that "the term significance, as used by the statistician, has a somewhat specialised meaning," and gave certain numerical illustrations, concluding with the words: "Evidently each must judge for himself what limit of probability to take as a standard, but it is well to remember that such standard is, in some measure, arbitrary."[12] Assuming that Sir Almroth did not consider these passages sufficiently clear, it is strange that he did not at least give his readers an opportunity of forming their own opinions. After all, and I hope there is no undue bitterness in the remark, it is rather a grave charge to bring against scientific writers that they have attempted to make capital out of the ambiguity of common words. Such a charge ought not to be insinuated without good grounds. I hope to have demonstrated that Sir Almroth Wright had no grounds whatsoever, good or bad, for the accusation.

I now turn to a more important matter. After dealing with the technical difficulties of opsonic estimations, Sir Almroth Wright says:—

> But the principle which holds of the hæmocytometrical will also hold in connexion with the opsonic count. There is no logical principle to compel anyone to pay a ransom for the incompetence of others by himself counting more cells or squares.
>
> In the opinion of the mathematical statistician all this will be beside the mark. "Have I not"—thus, no doubt, will run his mental comment—"specially gone out of my way to guard against such absurdity being imputed to me by basing my computation of the number of leucocytes which require to be counted upon data in which the functional error of the operator has been brought down to its absolutely irreducible minimum?"
>
> The good intention may be very freely conceded, but the statistician has perhaps overlooked the fact that he does not, in his capacity as an expert with figures, possess—and his record proclaims this truth aloud—any greater qualification for deciding what is good or bad technique than for deciding what measure of probability may safely be taken as a guide in practical medicine.[13]

Once more we have to remember the probable knowledge of the ordinary medical reader. Would he be likely to infer from these remarks: (1) that our first paper was based entirely upon material supplied from St. Mary's Hospital, part of it both prepared and enumerated, the whole of it prepared in the very *sanctum sanctorum* of the opsonic ritual? (2) that two independent examinations (Pearson's and our own) had failed to reveal any essential difference between the material prepared outside and that prepared inside St. Mary's?

It is, of course, conceivable that the passage I have quoted is Sir Almroth Wright's way of intimating that he has grave doubts as to the adequacy of the St. Mary's technique, but if this be so, he should really have told us whom we ought to trust in these matters. The conclusion which I invite the reader to adopt is that Sir Almroth Wright's principal accusations consist of more or less serious misrepresentations of what the statisticians really did. This seems to be due to a very superficial examination of the work stigmatised, and suggests that Sir Almroth Wright has either read the papers in question carelessly or is unable to comprehend them. The adoption of either alternative goes far to discredit the claim he, in effect, makes to sit in judgment upon the applicability of statistical methods to medical problems. It is true that in the last sentence quoted Sir Almroth Wright raises the question of the degree of probability or improbability which should be adopted in deciding medical points, but as a decision on this matter presupposes the applicability of the statistical method by which the probabilities are enumerated, it can hardly be raised seriously in view of Sir Almroth Wright's repudiation of the method.

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[12] The Practitioner, May, 1908, p. 8, of reprint.

[13] Wright: op. cit., p. 165.

Whatever view the reader may form as to the justice of my conclusions, he will certainly agree that this examination of the opsonic index controversy is a necessary prelude to a study of Sir Almroth Wright's scientific principia.[14] Statistical researches are laborious in execution, and even the perusal of their results entails some degree of attention. Many people have, I am sure, been relieved to hear on Sir Almroth Wright's authority that the technical processes evolved by biometricians have no value in medical inquiries. It may not be amiss to quote a few sentences of Macaulay's criticism of the utilitarians :—

> We apprehend that many of them are persons who, having read little or nothing, are delighted to be rescued from the sense of their own inferiority by some teacher who assures them that the studies which they have neglected are of no value, puts five or six phrases into their mouths, lends them an old number of the *Westminster Review*, and in a month transforms them into philosophers.

If there be any in medical circles disposed to welcome a teacher who contemns statistical reasoning, they should consider whether his own competence in the subject is beyond criticism.

We may now pass to the examination of Sir Almroth Wright's finally matured process of inquiry, his new medical organon.

II. *Sir Almroth Wright's Analysis of Scientific Methods.*

I must premise this section with the remark that, although I frequently cite the actual words of Sir Almroth Wright, it is difficult to give an accurate summary of another's reasoning in the case of a controversial topic like the present. The reader is asked to check my summaries by comparing them with the original statements.

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[14] It will also be remembered that the *quantitative* nature of the opsonic method has often been contrasted by its admirers with the qualitative and "authoritative" methods of the ordinary clinician.

Sir Almroth Wright reduces the methods at our disposal to two genera, Crucial Experiments and Cumulative Experiments. The characteristics of a crucial experiment are said to be "(*a*) The experiment can be instituted only when perfectly simple experimental conditions have been arrived at; (*b*) we obtain, on repeating the experiment, always the same result; (*c*) the conclusion which emerges is a categorical or universal truth—i.e., a truth which holds good everywhere where the conditions which are laid down for the experiments are realised." Since this method is not further discussed, I may interpolate here the few observations I desire to make.

Sir Almroth Wright does not, I think, realise that the fundamental distinction attempted to be made by him between this and other methods is illusory, and has overlooked the force of his own words under (*b*) (*supra*). One naturally asks *how many* repetitions of the experiment justify the assertion that (*b*) *has* been fulfilled? The anti-statistician who considers this point will have some misgivings lest after all the application of a canon of statistical inference be involved. As a sometime demonstrator of physiology I may cite a familiar "crucial" experiment. Three test tubes are prepared (A, B, and C). A contains fibrin, glycerin extract of pig's stomach, and water; B contains the same ingredients except that 0·2 per cent. solution of hydrochloric acid is substituted for the water; C is the same as B except for the omission of the gastric extract. The three tubes are incubated at body temperature for, say, half an hour, and at the end the student notes that the fibrin has partly disappeared from B and is unchanged in A and C. The student is expected to draw from this formal experimentum crucis the conclusion that the presence of mineral acid is essential for the performance of proteolytic action by gastric extract. Since the experiment has been repeated countless times the conclusion is logically warranted, but in truth the conclusion is not really drawn from the individual experiment, which is strictly more of the nature of a demonstration than a proof. Let us place ourselves in the position of the physiologist who, moved, let us say, by some speculation, first performed the experiment, would he have been justified in straightway proclaiming that he had *proved* the point? Suppose some serious therapeutic issue turned on an answer to the question, Is gastric proteolysis possible in the absence of mineral acid? would he be justified in taking action upon the strength of a single crucial experiment (assuming even that all his materials were chemically pure)? The answer is, of course, No, and Sir Almroth Wright recognises this by his condition (*b*).

We find ourselves, therefore, compelled to evolve some logical process in virtue of which we can ascertain how nearly, in any given case, the condition (*b*) has been fulfilled. Were Sir Almroth Wright better acquainted with the history of mathematical statistics, he would know that a problem closely akin to this one is a classic of the science. The matter is of interest as an example of Sir Almroth Wright's incomplete grasp of the principles he essays to describe, but is not otherwise germane to the discussion, and we may pass on to the subject of cumulative experiments.

> We fall back upon the *method of cumulative experiments* when we have to experiment in conditions which cannot be sufficiently simplified to allow of the institution of a crucial experiment. When this is the position of affairs we can no longer expect to obtain on repeating our experiments always the same result. And we here can no longer hope to deduce a law from the data of a single experiment,[15] or to formulate a thesis which will hold true in every individual case and of every individual in a class. Conclusions which will be of value can, however, still be reached by marshalling and evaluating the data of a series of substantive experiments and a series of control experiments, and comparing these together. These data may be marshalled and evaluated either (*a*) by the *experiential method*, or (*b*) by the *statistical method*.

The characteristics of the *experiential method* are that—

> We take into account the whole complex of impressions which have been left upon the mind by experience, we arrive at a *generalisation* (which is the general law or general evaluation of the class). This will express the result which we have witnessed in the majority of our cases; and it will, if our experience is a typical one, hold good of a majority of every other series of such cases.

This method is said to be one of "unrestricted outlook," every feature of the case or experiment having its proper share in forming the impression left upon the mind. Its application presupposes a high degree of skill upon the part of the experimenter and reasoner who are, and must be, one and the same person. All great advances in medicine, such as the employment of quinine in malarial diseases, have been arrived at by the employment of this method, and the rules as to what conclusions obtained by this process are valid reduce to the following :—

> (*a*) *Experiential judgments with respect to which there is among observers a serious difference of opinion need not be accepted.*
>
> (*b*) *Experiential judgments to which every qualified observer subscribes must be accepted.*

The expressions "serious difference of opinion" and "qualified observer" are neither defined nor illustrated, but with respect to the former term, it would appear that what is required is a "consensus, or a practical consensus of expert opinion" and with regard to the latter that "qualified" = "expert."

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[15] It would be inferred from this sentence that we may sometimes hope to deduce a law from the result of a single experiment. It would be interesting to know when, as Sir Almroth Wright has expressly told us that a crucial experiment needs repetition. (M. G.)

Again

> Where there is a conflict of opinion between observers the proper course to pursue is to appraise the relative weight of the authorities who are ranged over against each other.

In certain cases, a unanimity of expert opinion has supported an incorrect conclusion—e.g., the universal vogue of blood-letting. This was due to neglect of an essential part of the experiential method—viz., the institution of control experiments.

The *statistical method* involves three operations.

1. Critical study of the raw material.

> We here endeavour to discover in our cases a criterion upon which to build up our statistics. This criterion may be either (*a*) a really *critical feature* (the fact of escaping infection, or of recovering from attack would, for instance, constitute such a critical feature) by reference to which our substantive and control cases can be distributed into two sharply contrasted sub-groups of pluses and minuses, the individual cases being represented by a plus or a minus according as they do, or do not, exhibit the critical character; or (*b*) a *feature* which is common to all the cases, which can be expressed in figures and which *varies in a significant manner* (let us say such a feature as the number of days during which each patient's temperature has ranged above the normal).

2. Sifting the raw material.

> The basis upon which we are to go to work having been settled, we construct upon that basis a skeleton framework for our tables, and enter each substantive and control experiment as it comes in its proper column, putting out of sight and regard every element in the case other than the feature we have elected to consider.

3. Evaluation of the results.

> We count up the pluses and minuses and find the relative proportion of these in our series of substantive and control cases; or, as the case may be, we total up the figures which represent the respective values of the significant feature in each individual case, and make a comparison between the totals for our two groups of cases. In either case we express the final result in the form of a percentage; and, where the occasion calls for this, compute, or have computed for us, the "probable error" which we should be incurring if we took the substantive and control cases upon which we based our calculations as absolutely average samples of the classes they represent.

The difficulties of this method lie in the discovery of a really critical feature. Thus, in the case of phthisis, we have several possible criteria—e.g., the disappearance of bacilli from the sputum, a gain of body weight, the disappearance of pyrexia, &c. It might be suggested that we could obviate the difficulty by "allotting a certain arbitrary number of marks to each significant clinical phenomenon and then evaluating in accordance with such convention."

But such a scheme would resolve itself into—

> A proposal to make a number of separate statistical tables, and to combine the results in such a way as to make the statistical method a method of unrestricted outlook.

This proposal would

> Make shipwreck upon the fact that it would be impossible to include every case in every column of such a compound statistical table (the apyrexic cases, for instance, would have to be omitted from the column relating to pyrexia). And, again, it would make shipwreck upon the fact that there would be no accepted rate of exchange at which improvement in one respect could be reckoned against improvement in another; no basis for setting off, let us say, a temporary gain in weight in one patient against a temporary reduced pyrexia in another.

The statistical method also requires a much larger number of cases than the experiential, since the latter takes account of every feature in each case, the former only of some features. Since the statistical method is one of restricted outlook, requires a very large number of cases, and employs a "quite arbitrary scale of values," it has no theoretical advantages over the experiential method. It has also serious practical drawbacks. Thus it encourages ill-trained workers to fancy that their results, if dealt with statistically, are of value equal or superior to those of trained experts who do not adopt such methods, and leads to the vogue of "the armchair statistician who claims to sit as judge of appeal upon all evaluations of cumulative experiments." We can, therefore, "afford to let all this laborious ineffective striving after the accurate evaluation of cumulative experiments go."

I think the preceding paragraphs contain a substantially accurate account of Sir Almroth Wright's doctrine (although I would again urge the reader to consult the original), and may attempt an examination of it, beginning with his exposition of the statistical method. A word of explanation is, however, due to my biometric colleagues who will protest that such a wealth of quotations was hardly necessary. I must point out, in the first place, that the ordinary educated man untrained in statistical methods must needs be the final arbiter of the merits of the controversy, and in the second place that I have no right to expect even my co-workers to take my word as to the real extent of Sir Almroth Wright's knowledge of statistics without producing documentary evidence. The following paragraphs are intended rather for the general reader than for the trained statistician.

The average reader is therefore informed that statisticians do not necessarily express their final results "in the form of a percentage," nor do they, when several variables are in question, adopt a system of "allotting a certain number of arbitrary marks to each." Thus, suppose we are anxious to measure the respective shares of different possible factors of the rate of infant mortality. It has been said, for instance, that (1) the method of feeding, (2) the fertility rate, and (3) the poverty of the parents are all of importance. In endeavouring to evaluate the relation between changes in these last three variables and changes in the first, the statistician connects changes in the first with changes in all the others in a single equation, called a multiple regression equation. Each term of this equation measures in the appropriate unit (not arbitrarily chosen, but dependent upon the nature of the quantity and its variability) the effect of varying the character in question.[16] In a precisely similar way, the number of deaths from plague which occur in a village depends, *inter alia*, upon the number of the inhabitants and the length of exposure to infection. The relative importance of these two factors is determined as before.[17] A similar problem is to ascertain the relative importance of size and proximity to means of communication, such as railways, upon liability to suffer from an epidemic of plague.[18] All these examples refer to cases in which the variables can be expressed in terms of a quantitative scale: similar but not identical considerations apply to cases where this is not possible. An instance is afforded by the question, Does the apparently greater chance a well-vaccinated person enjoys of passing successfully through an attack of small-pox depend upon an association between economic or social well-being and efficient vaccination? This problem has also been dealt with by the method of correlation.[19] Indeed, it might be said that such dispute as exists among statisticians with regard to the treatment of non-quantitative problems is not as to the general method, but as to which of its various particular forms should be employed in any given case.

I have cited a few of the first examples which occurred to me; many others might be given, but it is vain to multiply instances in refutation of a charge which the most cursory examination of statistical journals, or even of an elementary text-book, proves to be ridiculous. To say more on this might give point to the scornful question--

.... usque adeone
scire tuum nihil est, nisi te scire hoc sciat alter?

We accordingly reach the result that the "restricted outlook" of the statistical method exists only in Sir Almroth Wright's imagination.

We next pass to the charge that the statistician supposes or suggests that the adoption of his method places workers of varying technical skill upon an equality. I may point out that this accusation was, in effect, preferred against White and myself in connexion with the opsonic inquiry. I have already put before the reader as fair an account as I could of that controversy, and will merely ask the following questions.

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[16] Greenwood and Brown: Journal of Hygiene, vol. xii., 1912, p. 5.

[17] Greenwood: Ibid., vol. xi., 1911 (Supplement), p. 62.

[18] Greenwood: Ibid., p. 47.

[19] Macdonell: Biometrika, vol. i., p. 375, and vol. ii., p. 135.

1. Does Sir Almroth Wright suppose that the astronomical observations of "experts" and amateurs are of equal value?

2. Does he suppose that Gauss believed the adoption of his methods of measuring errors of observation and personal equation put all observers on an equality?

3. Do modern astronomers consider that they can "afford to let all this laborious ineffective striving after the accurate evaluation of cumulative experiments go"?

The rôle of authority in science is, however, far too important a point to be passed over without careful consideration, and it is in this connexion that Sir Almroth Wright brings his most effective *ad captandum* charge against the statistician. In these days most educated men have some idea of the elaborate technic, the familiarity with the use of complex apparatus, the patience and the acumen which go to produce even a small piece of bacteriological work. We may go further and assert that many people have vivid experience of some sudden attack of illness in themselves or one of their families. A skilled medical man has been called in, has examined the patient, and pronounced a judgment which has been verified to the last detail by subsequent events. Perhaps they have questioned him as to his reasons and the replies have seemed trivial or unintelligible. Yet the event has borne out the expert's judgment. To one having passed through such an experience the trained medical expert must indeed seem the hero—

> Cuncta videns magno curarum fluctuat aestu;
> Atque animum nunc huc celerem, nunc dividit illuc,
> In partisque rapit varias perque omnia versat—

and the suggestion that some outsider who has never perhaps entered the walls of a bacteriological laboratory, or treated the simplest of complaints, should presume to say whether a medical expert is or is not justified in the enunciation of a particular conclusion must seem (as in truth it is) monstrous.

But the nature of the illustration just outlined will suggest an important distinction. It is the distinction between grounds of *proof* and grounds of *action*. Let me try to explain this. I am, let us suppose, a man of education, but no medical training, and a child of mine is taken ill with abdominal symptoms. I call in my family medical attendant and he says: "I think this is a case of appendicitis requiring surgical treatment, and I advise you to have a surgeon to consult with me." I of course agree and he recommends X. X examines the patient and says: "This is a case of acute appendicitis. I must operate at once or I cannot answer for the consequences." Perhaps I ask for more information, and he says: "There is pyrexia, muscular rigidity, tenderness at McBurney's point, nausea, and vomiting; further, your medical attendant tells me that the child has had a similar but milder attack before, and experience teaches me that in the case of second attacks immediate operation is the proper course." Most of this will be entirely unintelligible to me, but I shall reflect that (1) my medical man, who has always seemed an able man, advised me to consult X ; (2) I have heard X well spoken of by people I could trust ; (3) I do not follow X's reasoning, but if he is right, and I do not let him do what he proposes, the child will perhaps die. The conclusion emerges that I ought to follow X's advice—that is to say, the competence of X, the only matter respecting which I have evidence, is sufficiently established to form a ground of action for me.

The state of affairs is very different if the question be no longer what should be done in a specific case, but what is the cogency of the reasons which justified X's action in that case. Experts are agreed that not all patients with the signs and symptoms enumerated have really got appendicitis, and that not all victims of a second attack of appendicitis die when untreated. It is not *certain*, therefore, that X was correct. How are we to estimate the probability of his being so? Suppose that X obstinately confines himself to the argument that his experience and that of fellow experts prove that his opinion would be correct in the vast majority of such cases, then the non-medical scientific man is permanently deprived of any ground of *proof*. It is likely, is indeed of everyday occurrence, that he will now fall into the error, mentioned by Sir Almroth Wright, of comparing things not *in pari materia*. He will note the utterances of X with respect to other matters, say votes for women, and if he finds that X talks nonsense or draws illogical inferences with respect to that question, the *materia* of which is non-medical, may hastily conclude that X's surgical inferences are not better founded. But not only is the non-expert destitute of valid grounds of proof; the expert is in hardly better case. He cannot be sure that he attaches the same meaning to the words "great majority of cases," "consensus of opinion," "teaching of experience," as another expert. He is unable to fulfil the requirement of a valid scientific inference—viz., that it is such as "could be drawn by every logically trained normal mind if it were in possession of the conceptions upon which the inference has been based."[20] Are we, then, to conclude that the probability of X's opinion being correct cannot be made the subject of scientific investigation? The fact that surgeons are in the habit of publishing statistics of results would seem to suggest that such is not their own opinion. But from the moment this course is adopted the

[20] Pearson's Grammar of Science, Part I., p. 56.

attitude of the non-medical seeker after truth is altered. He may, perhaps, allow that the selection of *materia*, whether it be or be not made upon good grounds, is beyond his judgment, but he will hardly bear to be told that one mistaken diagnosis in twenty is equivalent to certainty, and that any hesitation to admit this is tantamount to permitting the armchair statistician to lay down the law respecting medical problems. We now see the fundamental confusion of thought which prevails as to grounds of proof and grounds of action in some medical minds. If it be said that a twenty to one chance in his favour was an adequate ground of action to X and that such a grade of probability is the highest which can be expected to occur in practical medicine, no immediate reply can be given. An answer to the first part of the question depends upon an evaluation of the consequences which may be expected to follow upon the doing or not doing that which depends upon the correctness of the conclusion. The second part necessitates an inquiry as to the grade of probability in medicine which does in fact pass for certainty. For instance, is the probability of a patient with unrelieved strangulated hernia dying about 20 to 1 or more? These fanciful illustrations will have served their turn if they have suggested that the sphere of medical authority is in need of definition. In particular, it is plain that we should be vouchsafed more light upon the questions as to how a "difference of opinion" is ascertained to be "serious" when a majority becomes a "practical consensus," and just how far the "qualifications" of a medical expert extend. It is disappointing to find that a qualified arch-expert like Sir Almroth Wright has so little to tell us on these points. Perhaps, however, a discussion of them is reserved for another paper, which will also explain how the non-expert proceeds to "appraise the relative weight of the authorities who are ranged over against each other." But, it will be said, does this leave the withers of the statistical "expert" unwrung? Has not a distinguished epidemiologist spoken despairingly of an attempt to "decipher the meaning lying hidden behind the hieroglyphics constituting the chief means of expression in the literature (if we may venture to call it such) of the biometricians"? The answer is sufficiently simple. The biometrician, *qua* biometrician, ***discloses the data upon*** *which his conclusions rest, the methods by which he has analysed them, and the conclusions which, in his opinion, may* ***be drawn***. He does not appeal to the experience of statistical experts, nor found conclusions upon a (practical) consensus of (qualified) opinion. It is open to any reader to study his paper or not to study it, to decipher the hieroglyphics or not to decipher them. To gibe at the assumption of authority by statisticians because one has not troubled to master their notation would be as reasonable as to charge v. Kries with attempting to set up as a physiological dictator, and to promulgate dogmas because he writes in German, if the critic does not understand that language. There is a little more to be said on this matter of statistical authority, but it can be reserved for a later section. The general bearing of Sir Almroth Wright's charges against the statisticians has been, I hope, elucidated, and I submit to the reader's favourable notice the following propositions.

1. Sir Almroth Wright's analysis of the statistical method is imperfect, and based upon ignorance of current biometric work, as set forth in scientific journals and text-books.

2. The claim of the medical expert that he alone is competent to say what his experiments do or do not prove is not merely unscientific, but anti-scientific. The *prima facie* reasonableness of such a claim is due to a confusion between grounds of action and grounds of proof.

3. It is of the essence of science to disclose both the data upon which a conclusion is based and the methods by which the conclusion is attained. No observer who fulfils these conditions can be accused of unwarrantably assuming authority.

4. The requirements of the last proposition are fulfilled by those biometricians who have investigated medical problems and Sir Almroth Wright's charges against them are consequently unjust.

We are now in a position to inquire whether the experiential method as described by Sir Almroth Wright is a trustworthy instrument of research.

The "Experiential" Method.

The essence of the experiential method is that *all* the significant features of every case have been taken into account by the expert observer, "we can feel confident that no significant factor has been excluded from consideration, and that, as far as possible, credit has been given for every such factor." The method is again particularised in the following terms:—

> When we employ it we let the two separate streams of experience which correspond to the twin series of substantive and control experiments filter through our minds and then compare the impressions which have been imprinted. While no complete account can be given of the psychological processes by which this comparison is carried out the two following points may be noted.
>
> When we bring into application the experiential method we take into consideration every feature of each case, and not, as in the case of the statistical method, only one selected feature—in other words, the *experiential* is a method of *unrestricted;* the *statistical* a method of *restricted outlook.* And the mental record upon which we proceed need not be inaccurately kept or blurred. When, for instance, we obtain in a succession of consecutive cases one and the same result, each succeeding case will render more distinct the impression made by the preceding case; and when a case which is at variance with a series of previous cases turns up, it will, by contrast, stand out very clearly in our consciousness.

It would be doing Sir Almroth Wright an injustice to suppose that his remarks are to be taken quite literally. If they were so taken, we should have to believe that the experiential method can only be used by prophets, since it is often impossible for anyone else at an early stage of a research to say precisely what are and what are not significant features of the results. Thus, most people would agree with the following remarks of Schopenhauer :—

> Any kind of important book should immediately be read twice, partly because one grasps the matter in its entirety the second time, and only really understands the beginning when the end is known ; and partly because in reading it the second time one's temper and mood are different, so that one gets another impression ; it may be that one sees the matter in another light.

Sir Almroth Wright probably means that in any research there will be so many points of similarity to some previous research that an expert is likely to recognise the significant features which present themselves. But even if we rationalise his words in this way, the difficulties by no means vanish. We have to take account of the concluding phrases in the passage cited from Schopenhauer. What is our warrant that the recording machine is constant ?

It is the experience of everyday life that the same external stimulus does not always call up the same sensation, and that different stimuli may elicit an identical response. What alchemy of medical experience can free the expert in that science from the ordinary weakness ? Surely it is the universal recognition of this which makes a disclosure of the evidence a first principle in non-medical scientific work ? We all admit that in any complex of sense impressions there may be some which it is difficult or impossible to place on record, but the fact that we have not within ourselves any constant standard of measuring these fleeting impressions deprives them of logical validity. Of those who trust to such methods we may ask with Berkeley, "Whether men may properly be said to proceed in a scientific method, without clearly conceiving the object they are conversant about, the end proposed, and the method by which it is pursued ?"

But, it may be said, the significant features in medical experience, the criteria, are always or mainly such as cannot be characterised in words or, *a fortiori*, invested with quantitative significance. But Sir Almroth Wright himself bears witness against this. Each of the criteria which he instances in the case of phthisis can readily be so expressed. He gives (1) disappearance of bacilli from the sputum ; (2) disappearance of pyrexia ; (3) gain of body weight ; (4) return of patient to full work. If we add (5) adoption or neglect of a certain treatment, it is plain that, in theory, we could, given an accurate written record, obtain a mathematical function expressing the relations subsisting between all the variables. We have, of course, no sort of right to say that the record provided in such a case by the non-expert is of equal value with that furnished by the trained observer; but we have every right to call upon the trained observer to put his records before us. The data are evidently not of so unsubstantial a nature that we have a right to substitute for them the impression they produced at the moment of compilation, since we have seen that such impression is produced upon a recording mechanism responding unequally at different times. We therefore conclude (1) that the so-called method is not in logical strictness a scientific procedure at all, since the results not only may vary from person to person but may be inconstant in the same observer at different times; and (2) that, Sir Almroth Wright himself being the judge, the significant features of a medical experiment are by no means of a character which precludes the employment of methods which involve a disclosure of the grounds of proof.

But little more remains to be said respecting Sir Almroth Wright's methodology. We have only to refer to his allegation that the so-called experiential method has justified itself by results. He cites (1) the employment of sulphur ointment as a remedy for itch; (2) the use of quinine in malaria; (3) the use of salvarsan in syphilis; and (4) the employment of strepto- and staphylo-coccus vaccines in localised infections due to those organisms.

With respect to (1), as Sir Almroth Wright ranks it among the instances of the method of crucial experiments, I need merely refer to my criticisms of his analysis of that method. As to (3) and (4), they belong to recent history, and the many writers who have supported their conclusions by statistics may be allowed to say for themselves whether they regard their statistics as merely decorative. The case of quinine in malarial infections is, however, not a recent fact. If the general reader consults the works cited below[21] he will find that quinine obtained a permanent place in the therapeutic armamentarium in days when, as Sir Almroth Wright remarks, the employment of a series of control experiments was unheeded. Consequently, if we commit—and Sir Almroth Wright says we do commit—the fallacy of comparing things not *in pari materia*, when we adduce the former unanimity of opinion as to blood-letting as an instance of the danger of accepting a consensus of expert opinion as a guide to truth, Sir Almroth Wright himself commits it when he brings the case of quinine to support his proposition. Naturally, if we marked the successes and neglected the failures, it would be easy to show that random guessing is a sound instrument of inquiry.

This brings me to the conclusion of my analysis of Sir

[21] Haeser: Lehrbuch der Geschichte der Medicin, vol. ii., p. 422. See also Creighton: History of Epidemics in Britain, vol. ii., p. 320.

Almroth Wright's contributions to the theory of scientific method, and I cannot do better than express my judgment in a phrase adapted from his own picturesque and forceful vocabulary. I think "we can, therefore, afford to let all this laborious ineffective striving after" the rehabilitation of dogma and expert infallibility go. We must frankly recognise that medicine, in so far as it is a science, is *in pari materia* with every other science, and we must repeat with Karl Pearson that "the day of authority in any branch of science *has* gone by."

I have now, to the best of my ability, submitted the case as I conceive it, to the judgment of the scientific reader. But I recognise that an effective attack is much easier than a defence, and that Sir Almroth Wright is not, perhaps, the kind of writer whom it is least easy to assail. I do not doubt that he has published his remarks in the hope of raising the scientific standard in our common profession, and that he attaches more importance to his constructive work than to his destructive criticism. Animated by his example, I should like to be allowed to devote the concluding passages of this paper to an attempt at indicating the constructive reformation which seems to me needful for the advancement of medical science.

The Method by which a Therapeutic Problem ought to be Investigated.

Let us suppose that the question is whether a certain treatment is of advantage in acute lobar pneumonia. We must first inquire whether the morbid state connoted by the phrase "acute lobar pneumonia" is clinically recognisable. The question is answered in the words of Sir William Osler: "No disease is more readily recognised in a large majority of cases. The external characters, the sputum, and the physical signs combine to make one of the clearest of clinical pictures. The ordinary lobar pneumonia of adults is rarely overlooked."[22]

The next point to be investigated is the variation of fatality in cases not treated by the method under investigation.

(*a*) *Influence of age.*—That the fatality increases with the age of the patient is well known and evidence need not be quoted here. Naturally, in comparing fatalities it will be necessary to correct for age.

(*b*) *Sex.*—The influence of sex is not so marked, but allowance can similarly be made for it.

(*c*) *Secular variations.*—It would appear that these are of minor importance. The following tables contain some evidence on this point.

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[22] The Principles and Practice of Medicine, eighth edition, 1912, p. 97.

*London Hospital Experience of Pneumonia in Persons over 10.**

Period.	Cases.	Corrected fatality rate.
1784-1800	235	23·9 per cent.
1814-30	184	31·6 ,,
1831-53	152	21·4 ,,
1854-63	186	19·2 ,,
1864-73	222	22·9 ,,
1874-83	386	26·0 ,,
1884-93	1381	20·1 ,,
1894-1903	1804	23·4 ,,

It also appears that the fatality of hospital cases from different institutions in the same country during the same period varies but little. Thus we have :—

Source of data.	Number of cases (persons over 10).	Corrected fatality rate.
St. Mary's and Charing Cross Hospitals (1897-1906)	647	21·6 per cent.
Guy's Hospital (1902-06)	959	23·5 ,,
St. Thomas's Hospital (1880-90)	442	23·4 ,,
Glasgow Western Infirmary (1900-10)	582	21·4 ,,
London Hospital (1894-1903)	1804	23·4 ,,

* The data upon which these tables are based have been fully analysed in a paper by Lieutenant Candy and the present writer (Journal of Royal Statistical Society, vol. lxxiv., 1911, p. 365).

(*d*) *The influence of social class.*—Evidence capable of being analysed has been sparingly published. The 873 cases recorded by the British Medical Association's Collective Investigation Committee in 1886 show a corrected fatality rate of 17 per cent., which is below the London Hospital rate for the same period. The results of Huss[23] at Stockholm, more than 40 years ago, suggest that the fatality in the Military Hospital was about 7/11ths of the rate obtaining in the General Hospital.

(*e*) *Influence of race or climate.*—We find striking differences in the hospital fatality rates of different countries, the rate at the Stockholm hospital in the "fifties" of last century being far below that recorded for the same period at Vienna or Basel.[24] There is a less striking difference between the recent London figures and those of Chatard from Baltimore.[25]

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[23] Huss: Die Behandlung der Lungenentzündung, &c., Anger's translation. Leipzig, 1861.

[24] Fismer: Deutsches Archiv für Klinische Medicin, Band xi., 1873, p. 391.

In view of what has been said, it will be plain that in comparing a series of treated cases with "general experience" attention will have to be paid to the differences noted, all of which can be tested by the statistical method. When a true control series is available, it will still be necessary to allow for race and environment. An inquiry into these points would seem a necessary prelude to an evaluation of the effects of any specific treatment.

These are all questions of great moment, and cannot be answered by appeal either to authority or to the introspective notions yielded by the "experiential method."

Having made due allowance for these difficulties, we shall proceed to compare the rate of mortality in the treated and untreated cases. This will involve a careful sifting of the material, since we must reject such cases as died in consequence of some accident in no way connected with the evolution of the disease. The criteria of exclusion must be defined, and no case excluded without the grounds of such exclusion being clearly stated and the particulars published in full to give others an opportunity of judging the sufficiency of the criterion.

Next, we shall in some cases be able to compare the percentages and determine the probability that such difference as results might be an "error of random sampling." This will by no means complete the task, however, since it might happen that the treatment, although not associated with a significant reduction of fatality, did influence the course of the disease. The features which it is desired to measure having been determined on, we can by the method of multiple correlation endeavour to connect the variations of such features with each other and with those of the therapeutic factor we are studying. Since in general it will be difficult to secure controls and treated samples absolutely alike in other respects, the method of correlation is likely to be required in most cases. We shall, indeed, be fortunate if we are able to "express the final result in the form of a percentage."

I have outlined the process by which, as I think, such a problem may be investigated. The essence of the whole matter is to ask ourselves at every turn—Is the control a real control? What is the probability that such and such an event is due to such and such a cause? There is no intrinsic merit in numbers and percentages or in coefficients of correlation, their value is in aiding us to think clearly and compelling us to express conclusions in a language which all may master if they choose. But the reader will detect the weak point, covered by the words, *if they choose.* At

[25] Chatard: Johns Hopkins Hospital Reports, vol. xv., [illegible], p. [illegible]

the present time few choose to understand the language of statistics, and many attempt to excuse themselves by the assertion that a knowledge of the language is not attainable by the average medical man. This assertion is wide of the truth. During the past few years I have had in my laboratory several workers whose preliminary training in mathematics had been neither greater nor less than that of all qualified medical men. Any of these would bear me out in the statement that a knowledge of statistical processes sufficient to enable one intelligently to adopt the modern methods requires no more application and effort than are necessary in branches of investigation universally admitted to be within the competence of all post-graduate medical workers. I do not mean to suggest that the acquirements necessary for the original investigator in pure statistical theory can be conferred upon everyone. To assert that an ordinarily intelligent man can learn to play a fair game of chess does not mean that Morphys and Laskers are the product of environment alone. The analogy will indeed serve us a little more. In a complex position on the chess-board it may require a master to indicate the right continuation to enable, say, black to win. But the man with an ordinary knowledge of the game will be able to follow the line of play proposed by the master and to satisfy himself of its correctness, or the reverse ; he will ignore the confident assertions of those who do not even know the moves that no rational line of play can be laid down in such complex positions, that success is a matter of temperament or personal equation or "experience," &c.

The present statistical "experts" are in the position of the chess master and can afford to ignore the charge of seeking to set themselves up as scientific dictators made by those who do not know the moves. Naturally, if anyone arrogates to himself the rank of a chess master and proclaims that in any given position the right move is such and such, so many people have a fair knowledge of the game that he will soon be found out if he is a pretender. Unfortunately, this is not yet the case in medical statistics, and too much work is either ignorantly proclaimed to be a "mathematical proof" of something or ignorantly dismissed as "futile trifling." The cure for this state of affairs is *to learn the moves.* I rejoice to think that the cure is likely to be adopted, and it is pleasing for the advocates of medico-statistical methods to read the tribute of Sir William Osler paid in words which may fitly conclude this paper: "Karl Pearson's new iatro-mathematical school of medicine has done good work in making the profession more careful about its facts as well as its figures."

Lister Institute.

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ON ERRORS OF RANDOM SAMPLING IN CERTAIN CASES NOT SUITABLE FOR THE APPLICATION OF A "NORMAL" CURVE OF FREQUENCY.

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I.

Introduction.

Those who believe that the more closely a branch of knowledge adapts itself to the principles of quantitative reasoning, the more justly it merits to be ranked as a science, have been gratified by the improved standard adopted in the treatment of medico-statistical results. It is true that even now medico-statistical writers fall short of the attainments regarded as essential in some other departments of natural knowledge, and that a few prominent investigators vaguely denounce "mathematicians"—by which term is to be understood any one trained to employ modern biometric methods—as presumptuous intruders within the sphere of experimental medicine. Despite these obstacles, progress has been marked within recent years and we may have considerable confidence that future discussions as to the value of such procedures as vaccination or the determination of an opsonic index will be conducted with due regard to the claims of exact science.

It is, however, in the nature of things that a reform of this magnitude should be accompanied by certain disadvantages which tend to impede the march of ideas. For instance, reformers may urge that the employment of certain arguments requires for logical validity the application of some specific test. After much discussion, the point is conceded and then the test is in danger of being applied in other and unsuitable instances.

The particular illustration which has prompted these remarks is the employment of some consequences of the current theory of errors of random sampling in certain cases which frequently arise in medical and pathological work. At one time it was customary to base conclusions as to the efficacy of some method of treatment upon short series of cases without any statistical test being employed. A practitioner might find, for example, that of 100 cases of typhoid fever treated without any special precautions as to diet, six had died. Of a subsequent 100, dieted in a particular way, but two succumbed and a conclusion very favourable to the new method of treatment might be ventured. Owing to the partial permeation of medicine by quantitative methods, it is widely felt that this procedure is questionable and that the "probable error" of the result must be found. The medical writer who has attained this level accordingly refers to a text-book and tests his proportions upon the basis of a "normal" curve of errors with the binomial standard deviation $\sqrt{n\bar{p}\bar{q}}$.

The specialist in mathematical statistics is aware that the time-honoured theory of the "probable error" rests upon certain assumptions of a quite definite character not adequately fulfilled in the imaginary case described. Warnings as to this are given in the better text-books, and are indeed unnecessary for those who care to read the proofs of the usual formulae.

We must, however, bear in mind that not every medical man has either the time or the training requisite for the comprehension of mathematical analysis and many will be inclined to consult a book which, while giving formulae without proofs, contains explicit instructions as to their practical employment. Such a book as, for instance, Professor Davenport's *Statistical Methods*, seems admirably adapted to the needs of the laboratory worker. On p. 14 (2nd edition) he will find the following sentence:—"The probable error of the determination of any value gives the measure of unreliability of the determination; and it should always be found." The statement is commendably clear but, unfortunately, quite incorrect in many cases which come under the notice of the medical inquirer.

The present memoir is an attempt to make the limitations of the process recommended by Professor Davenport arithmetically obvious to the medical reader, and to provide the latter with some assistance in the exceptional cases. To the trained mathematician or biometrician I have nothing to offer which is novel and little which is of interest, while the medical reader may find some difficulties in following every step of the inquiry. I hope these difficulties have been reduced to a minimum, but a risk of falling between two stools has to be faced by any writer dealing with a subject not new in itself but relatively so in its applications. My biometric colleagues will recognise the difficulties of the task, and are alone competent to determine the measure of success or failure achieved.

II.

The chance of an event happening is $\bar{p}$ and of it failing, $\bar{q}$ ($\bar{p}+\bar{q}=1$). What is the "probable error" of $\bar{p}m$ successes in m trials?

In the problem stated the probability for the occurrence of an event and the independence of the happenings are supposed to be known. This *either* means that they have been ascertained by long experience *or* that their values (i.e. the value of $\bar{p}$ and the zero correlation between the results of successive trials) are defined by an hypothesis which we desire to verify. Accordingly the distribution of successes in m trials is given by the expansion of the binomial $(\bar{p}+\bar{q})^m$. If m be moderately large and $\bar{p} \sim \bar{q}$ small, the ordinates of a "normal" curve with Standard Deviation $\sqrt{m\bar{p}\bar{q}}$ are a close approximation to the terms of the binomial and the "probable error" of $m\bar{p}$ is $\pm\, {\cdot}67449\sqrt{m\bar{p}\bar{q}}$. This is the classical text-book case. Its limitations are obvious. If either $\bar{p}$ or $\bar{q}$ be very small unless m is very large indeed, and for all values of $\bar{p}$ and $\bar{q}$ when m is very small, the normal curve does not approximate closely to the binomial.

Consider this problem. A certain bacillus is stated to occur in the mouths of 2 per cent. of all normal persons. Twenty persons have been examined and the bacillus was isolated from two of them. Is this observation consistent with the truth of the hypothesis?

Let us find the chance that in 20 trials two or more successes would be met with if $\bar{p} = {\cdot}02$, $\bar{q} = {\cdot}98$.

By direct calculation we find this chance to be about 1 in 17. If we use a "normal" curve with Standard Deviation $\sqrt{20\,({\cdot}02 \times {\cdot}98)}$, the chance proves to be rather less than 1 in 25, or the probability determined in this way is only 68 per cent. of the real value. Of course when the number of trials is so small we could not expect a continuous function effectively to represent the binomial expansion, but even for m large the inadequacy of the "normal" curve, in the case of $\bar{p} \sim \bar{q}$ not small, must be insisted upon. I think the best way of making this clear arithmetically is from a consideration of the moment coefficients of the binomial $(\bar{p} + \bar{q})^m$.

With the ordinary notation we have:—

$$\mu_2 = c^2 m\,\bar{p}\bar{q},$$
$$\mu_3 = c^3 m\,\bar{p}\bar{q}\,(\bar{p} - \bar{q}),$$
$$\mu_4 = c^4 m\,\bar{p}\bar{q}\,\{1 + 3\,(m - 2)\,\bar{p}\bar{q}\},$$

and

$$\beta_1 = \frac{\mu_3^2}{\mu_2^3}, \quad \beta_2 = \frac{\mu_4}{\mu_2^2}.$$

In cases like the present, c may be taken as unity.

For a "normal" curve to be a good fit to the binomial, β_1 should be very small and β_2 nearly equal to 3.

Take as an illustration the values of β_1 and β_2 for different values of m where

$$\bar{p} = {\cdot}02, \quad \bar{q} = {\cdot}98.$$

We obtain:

m	β_1	β_2
100	·4702	3·4502
200	·2351	3·2251
300	·1567	3·1501
400	·1176	3·1126
500	·0940	3·0900
600	·0784	3·0750
700	·0672	3·0643
800	·0588	3·0563
900	·0522	3·0500
1000	·0470	3·0450

From these figures it is plain that even comparatively large values of m do not admit of the binomial being closely approximated to by a "normal" curve.

Since, however, direct evaluation of the terms of the binomial is very tedious when m is at all large, we need a curve which bears the same relation to the skew binomial that the normal curve does to the symmetrical binomial. Such a function was provided years ago by Pearson*, viz. his Skew Curve of Type III,

$$y = y_0 e^{-\gamma x}\left(1 + \frac{\gamma x}{s}\right)^s,$$

where
$$s = \frac{4}{\left(\frac{1}{m\bar{p}\bar{q}} - \frac{4}{m}\right)} - 1,$$

$$\gamma = \frac{2}{\bar{p} - \bar{q}} \quad \text{(taking the unit of measurement } c = 1 \text{ as before)},$$

$$y_0 = \frac{\gamma s^s \,.\, e^{-s}}{\Gamma(s+1)}.$$

To use this curve with the rapidity possible in the case of a "normal" curve, we need tables not at present published.

In any particular case, however, the curve may be calculated and the area between assigned ordinates approximated to with little labour.

To sum up, we have the following rules for practical work when $\bar{p}$ is known or assumed.

(1) When m is small, say less than 25, the binomial expansion should be directly evaluated.

(2) When m is moderately large and $\bar{p}$ or $\bar{q}$ not small, say not less than ·1, the ordinary method based on the "normal" curve can be trusted.

(3) If m is moderately large and $\bar{p}$ or $\bar{q}$ less than ·1, a skew curve of Type III should be fitted from the momental constants of the binomial and the areas between assigned ordinates estimated with the help of quadrature formulae.

III.

If in n trials an event happened p times and failed q times, what is the probable distribution of successes and failures in m subsequent trials and what are the respective chances of 0, 1, 2, ... m successes in m trials, it being assumed that the occurrences are independent and that the "universe" of events is indefinitely greater than $n + m$?

This problem is of fundamental importance. We note at once that when the last condition is imperfectly fulfilled an important special case may arise, for we then have:—

$\frac{n+m}{N}$ finite where N is the number of events comprising the "universe" or "population" from which the samples come. This problem of drawing from a "limited universe" will not be considered in the present memoir; it has been discussed in the paper of Pearson last cited*.

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* For a recent précis of the relevant facts, see Pearson, K. "On the Curves which are most suitable for describing the Frequency of Random Samples of a Population," *Biometrika*, 1906, Vol. v. p. 172.

The class of problem to which attention is now directed may be typified as follows:—

Fifteen "control" rats have been inoculated with a constant dose of a standard culture of plague bacilli and twelve succumbed in a certain time. Ten similar rats have been immunised by a method it is desired to test and five of these died after inoculation with a dose of culture similar to that employed upon the "controls." What is the probability that the deviation from the rate of mortality obtaining among the "controls" is a chance event?

Evidently the methods of pp. 69—72 cannot be used. To state that the *a priori* chance of dying is ·8 is to ignore the fact that the size of the "control" sample does not justify us in assuming that its proportional yield approximates at all closely to that of the whole population.

Let us, then, start from first principles, merely assuming (an assumption based on or supported by the fairly wide practical experience of civilised humanity) that all possible events are, *in the absence of any grounds for inference*, equally likely (Bayes' principle).

On this assumption, we have, by Bayes' Theorem for the chance P_x that the true probability of an event, observed to happen p and fail q times in n trials, is between x and $x + \delta x$:

$$P_x = \frac{x^p (1 - x)^q \, dx}{\int_0^1 x^p (1 - x)^q \, dx}.$$

A second trial of m being made, the total chance of its yielding r successes and $m - r$ failures is:

$$\frac{m!}{r!\,(m - r)!} \frac{\int_0^1 x^{p+r} (1 - x)^{q+m-r} \, dx}{\int_0^1 x^p (1 - x)^q \, dx} \quad \ldots\ldots\ldots\ldots\ldots\ldots (1).$$

This is, in modern notation, the result contained in the 7th of Condorcet's problems published in his *Essai*, 1785†, but Laplace had, eleven years previously, given the theorem with the omission of the term $\dfrac{m!}{r!\,(m - r)!}$ (i.e. working on the standard model of an urn from which balls are drawn, he assumed the drawings to have been made in an assigned order).

To Pearson‡, whose symbols will be used, belongs the credit of emphasising the enormous statistical value of the theorem. The usual method of treating (1) has been to show that, *under certain conditions*, the probabilities of different values of r can be represented by the ordinates of a "normal" curve*. The nature of the assumptions involved will be placed in the clearest light by the following considerations.

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* See Pearson, *op. cit.* pp. 173—5.

† See Todhunter's *History of the Theory of Probability*, p. 383, and for a similar result obtained by a different process in 1795 by Prevost and Lhuilier, *op. cit.* p. 453.

‡ Karl Pearson, "On the Influence of Past Experience on Future Expectation," *Philosophical Magazine*, 1907, p. 365.

Substituting 0, 1, 2, ... m for r successively in (1), reducing to B and then to Γ functions and finally evaluating each term, we have for the chances of 0, 1, 2, ... m successes in a sample of m after a first sample $n = p + q$:

$$C_0 \left\{1 + \frac{m\,(p+1)}{1\,!\,(q+m)} + \frac{m\,(m-1)\,(p+1)\,(p+2)}{2\,!\,(q+m)\,(q+m-1)} \dots\right\} \quad \dots\dots\dots\dots(2),$$

where
$$C_0 = \frac{\Gamma\,(q+m+1)\,\Gamma\,(n+2)}{\Gamma\,(q+1)\,\Gamma\,(n+m+2)}.$$

We may notice that, if p and q are both very large as compared with m, (2) reduces to

$$\left(\frac{q}{n}\right)^m \left(1 + \frac{mp}{q\,1\,!} + \frac{m\,(m-1)\,p^2}{2\,!\,q^2} + \text{etc.}\right)$$
$$= \left(\frac{q}{n}\right)^m \left(1 + \frac{p}{q}\right)^m = (\bar{p} + \bar{q})^m, \text{ where } \bar{p} = \frac{p}{n} \text{ and } \bar{q} = \frac{q}{n} \dots\dots(2)\ \textit{bis}.$$

The conditions for the approximation of this binomial to the "normal" curve have already been noted.

More directly, the approach of (2) to a "normal" form can be examined by treating the series in brackets, which is a hypergeometric series having as parameters

$$\alpha = -m, \quad \beta = p + 1, \quad \gamma = -(q+m), \quad \delta = 1,$$

by the method of moments and then noting the conditions under which the momental constants β_1 and β_2 approximate to the values 0 and 3 respectively. This method was adopted by Pearson who had, several years before the date of the publication last cited, obtained the moment coefficients of a hypergeometric series†.

The results are that:

$$\beta_1 = \frac{(q-p)^2}{m\,(p+1)\,(q+1)} \, \frac{\left(1 + 2\,\dfrac{(m-1)}{(n+4)}\right)^2}{1 + \dfrac{m-1}{n+3}} \dots\dots\dots\dots\dots\dots\dots\dots\dots\dots(3),$$

$$\beta_2 = 3\left(1 - \frac{2}{m}\right) \frac{1 + \dfrac{m-1}{n+4}\left(1 - \dfrac{8}{m-2} - \dfrac{9}{n+5}\right)}{1 + \dfrac{m-1}{n+3}}$$
$$+ \frac{(n+2)^2}{m\,(p+1)\,(q+1)} \, \frac{1 + 6\,\dfrac{m-1}{n+4}\left(1 + \dfrac{m-2}{n+5}\right)}{1 + \dfrac{m-1}{n+3}} \dots\dots(4).$$

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* See, for instance, Czuber's *Wahrscheinlichkeitsrechnung* (1903 Edition), pp. 151 etc.

† Karl Pearson, "On Certain Properties of the Hypergeometrical Series, and on the fitting of such Series to Observation Polygons in the Theory of Chance," *Philosophical Magazine*, 1899, p. 236.

If we write $\bar{p} = \frac{p}{n}$, $\bar{q} = \frac{q}{n}$, $\epsilon = \frac{\bar{p} - \bar{q}}{n + 2}$ and if m and n are both absolutely large, we find for (3) and (4)

$$\beta_1 = \frac{(\bar{q} - \bar{p})^2}{m(\bar{p} + \epsilon)(\bar{q} - \epsilon)} \frac{\left(1 + 2\frac{m}{n}\right)^2}{1 + \frac{m}{n}} \quad \text{........................(3 A).}$$

$$\beta_2 = 3 + \frac{1}{m(\bar{p} + \epsilon)(\bar{q} - \epsilon)} \frac{1 + 6\frac{m}{n}\left(1 + \frac{m}{n}\right)}{1 + \frac{m}{n}} \quad \text{...........(4 A).}$$

If now m be small *relatively to* n,

$$\beta_1 = \frac{(\bar{q} - \bar{p})^2}{m(\bar{p} + \epsilon)(\bar{q} - \epsilon)} \quad \text{and} \quad \beta_2 = 3 + \frac{1}{m(\bar{p} + \epsilon)(\bar{q} - \epsilon)} \quad \text{........(5).}$$

If n be small *relatively to* m,

$$\beta_1 = \frac{4(\bar{q} - \bar{p})^2}{n(\bar{p} + \epsilon)(\bar{q} - \epsilon)} \quad \text{and} \quad \beta_2 = 3 + \frac{6}{n(\bar{p} + \epsilon)(\bar{q} - \epsilon)} \text{...........(6).}$$

After exhibiting these results, Pearson remarks*: "Both forms result—for n or m large and the product of either with $\bar{p}$ and $\bar{q}$ not small—in $\beta_1 = 0$ and $\beta_2 = 3$, i.e. in the symmetry and mesokurtosis, which are for practical purposes closely enough represented by the Gaussian curve. But if m and n be commensurable, and either $\bar{p}$ or $\bar{q}$ moderately small, this result by no means follows."

It is accordingly plain that in *all* cases of m and n both small the use of a "normal" curve with S.D. $= \sqrt{m\bar{p}\bar{q}}$ is inappropriate. When $\bar{p} = \bar{q}$ the condition of mesokurtosis is fulfilled and the divergence from "normality" reduces itself to the difference between the Gaussian and Pearson Type II curves. The accompanying table illustrates this in a particular example.

A Second Sample of 10, *after a first Sample of* 100: $p = q = 50$.

Comparison of Series with Curves (Totals = 100).

Successes	Hypergeometric Series	Normal Curve S. D. $\sqrt{npq}$	Normal Curve S. D. $\sqrt{(n+1)pq}$	Curve of Type II $y = 2\cdot3412\left(1 - \frac{x^2}{63\cdot72999}\right)^{10\cdot2577}$
0	·146	·221	·333	·188
1	1·243	1·122	1·408	1·331
2	4·931	4·349	4·843	5·091
3	12·017	11·447	11·702	12·107
4	19·922	20·152	19·728	19·729
5	23·480	24·817	23·972	23·105
6	19·922	20·152	19·728	19·729
7	12·017	11·447	11·702	12·107
8	4·931	4·349	4·843	5·091
9	1·243	1·122	1·408	1·331
10	·146	·221	·333	·188

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* *Op. cit.* (1907), pp. 371—2.

A few arithmetical results may now be given.

Let $n = 100$ and $m = 50$.

	From Series (2)	Area * of "Normal" Curve, with S. D. $\sqrt{m\bar{p}q}$
$\bar{p} = \cdot 4,\quad \bar{q} = \cdot 6$		
Chance of 20—22 Successes	·255†	·271
$\bar{p} = \cdot 1,\quad \bar{q} = \cdot 9$		
Chance of 5—7 Successes	·3193	·4739
" 0—2 "	·1311	·1145
$\bar{p} = \cdot 01,\quad \bar{q} = \cdot 99$		
Chance of 0—2 Successes	·8938	·9202
" 3—5 "	·1007	·0022

We see how the liability to error increases with $\bar{p} \sim \bar{q}$.

An interesting special case may be discussed here which emphasizes the importance of the problem indicated.

Suppose the first sample has given all successes or all failures, so that $\bar{p}$ or $\bar{q} = 0$, how are we to measure its reliability?

Many unsophisticated users of formulae must have been puzzled by this case, since, construing the formulae *au pied de la lettre*, it would appear that after n successes in n trials, we ought to get m successes in m *with a probable error of* 0!

The paradox vanishes if we consider (2). Put in it $n = p$ and we have

$$\frac{m!\,(n+1)!}{(n+m+1)!}\left\{1 + \frac{n+1}{1!} + \frac{(n+1)(n+2)}{2!} + \text{etc.}\right\}\ldots\ldots\ldots\ldots\ldots(7).$$

From this we see that the ratio of the $(m+1)$th term to the whole sum (i.e. the chance of m successes in m trials) is $\frac{n+1}{n+m+1}$; from which we conclude:

(*a*) Only when n is very large as compared with m does the chance of obtaining 100 °/$_\circ$ successes in m trials approach unity.

(*b*) In particular if $n = m$ and both are large, the chance is about ·5.

For instance if we have had 100 °/$_\circ$ successes in 200 trials the chance of getting the same proportion in a subsequent 50 is about 4 to 1. If, on the other hand, $n = 50$ and $m = 200$ it is 1 to 4.

In view of what follows it may be worth noticing that a closed expression for the sum of any number of terms of (7) can readily be given.

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* Taking for area corresponding to x successes, the area between the ordinates $x - \cdot 5$ and $x + \cdot 5$.

† Approximate only, obtained by using Stirling's theorem in the expression

$$\frac{m!\,(p+r-1)!\,(q+m-r+1)!}{(m-r+1)!\,p!\,(q+m)!\,(r-1)!},$$

to find the rth term of series (2).

Write in Euler's identity[*]

$$1 - a_1 + a_1(1 - a_2) + a_1a_2(1 - a_3) + \dots + a_1a_2\dots a_n(1 - a_{n+1}) = 1 - a_1a_2a_3\dots a_{n+1},$$

$$a_1 = \frac{x}{y}, \quad a_2 = \frac{x + p_1}{y + p_1} \text{ etc.}$$

Multiply by $\dfrac{y}{y - x}$ and subsequently put $y = 0$.

We have
$$1 + \frac{x}{p_1} + \frac{x(x + p_1)}{p_1p_2} + \dots = \left(\frac{x + p_1}{p_1}\right)\left(\frac{x + p_2}{p_2}\right)\dots\left(\frac{x + p_n}{p_n}\right) \quad \dots\dots(8).$$

Putting
$$n + 1 = x, \quad p_1 = 1, \quad p_2 = 2, \dots p_m = m,$$

$$1 + \frac{n + 1}{1} + \frac{(n + 1)(n + 2)}{2\,!} + \dots = \prod_{y=1}^{y=m}\left(1 + \frac{n + 1}{y}\right) \quad \dots\dots\dots\dots(9).$$

Reverting to the general case, we note that for testing the divergence between first and second samples the formula (2) must always be employed when m and n are commensurable and $\bar{p} \sim \bar{q}$ not small. This rule certainly applies to all cases of m and n less than 300 or 400 and $\bar{p}$ (or $\bar{q}$) $< \cdot 1$. If m and n be large the best plan will be to fit to (2) the curve indicated by the momental constants, using its proportional areas (obtained by some convenient quadrature formula) precisely in the manner adopted with the tabled areas of the "normal" curve.

Such a method is, however, not convenient for laboratory workers nor specially appropriate when m is a small number, since in that case the terms of the discontinuous series are not closely represented by a continuous curve.

Evidently what one needs is a tabulation of the series (2) for different values of m, n and p.

Were it possible to obtain a simple formula for the sum of any assigned number of terms of (2), the computation of such a table would be a rapid process.

In the particular case $p = 0$ or n, such a formula has been given above. In the general case I have not reached any result[†] and more widely trained mathematicians, who have kindly allowed me to consult them, do not regard the problem as a simple one.

I therefore fell back upon the method of direct calculation. This is a straightforward but irksome task[‡].

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[*] See Chrystal's *Algebra*, Vol. II. p. 392, Ed. 1889.

[†] Formulae are available in certain types of Hypergeometric Series. *Vide* M. J. M. Hill, "On a Formula for the Sum of a Finite Number of Terms of the Hypergeometric Series when the Fourth Element is equal to Unity," *Proc. Lond. Math. Soc.* 1907, Series 2, Vol. V. p. 335; and 1908, Series 2, Vol. VI. p. 339. The methods of these papers cannot be used in the present case.

[‡] Sir Ronald Ross and Mr W. Stott have recently published ("Tables of Statistical Error," *Annals of Tropical Medicine and Parasitology*, Vol. V. No. 3, 1911) a set of tables for the use of laboratory workers. Their tables will be of great service in the cases in which p is not less say than 0·1, but are not, I think, available for the class of problem discussed in this paper, since they appear to be based on the "normal" theory of errors. It must be noticed that in an immense number of examples which arise in medical work p will not only be less than 0·1 but less than 0·01 (the prevalence of mental defect in children, albinism, epilepsy, etc. are instances), and for such cases the "normal" treatment is, as pointed out above, inappropriate and often misleading.

For the benefit of those who wish to extend my small tables, it may be worth indicating the arithmetical arrangement which I have found most convenient. I use the following scheme:

$n,\quad m,\quad p,\quad q,$	$\frac{1}{C_0}$
$q + m$	$100\, C_0$

Term		Multiplier	
1	(α)	$\frac{m\,(p+1)}{(q+m)\,1}$	(a)
$\frac{m\,(p+1)}{(q+m)}$	(β)	$\frac{(m-1)\,(p+2)}{(q+m-1)\,2}$	(b)
βb	(γ)	$\frac{(m-2)\,(p+3)}{(q+m-2)\,3}$	(c)
γc	(δ)	$\vdots$	(d)
$\vdots$	$\vdots$	$\vdots$	$\vdots$

The values of n, m, p, q and $q + m$ are written at the top of the sheet, $\frac{1}{C_0}$ and $100\, C_0$ are calculated and written in the right-hand top corner.

Two columns are next formed; the entries in the right-hand column having been made, any given term of the left-hand column is the product of the entries in the columns immediately above it. The entries in the left-hand column are added up and the sum checked by comparing it with $\frac{1}{C_0}$. Finally each term is converted into a percentage by multiplying with $100\, C_0$.

In this way, provided one has a mechanical calculator, a series having only a moderately large number of arithmetically significant terms is rapidly evaluated. Still, when all is said, the calculation of a table for values of m and n ranging from say five to a hundred and $\bar{p}$ from 0 to ·1 would need an heroic amount of patience. Even the present admittedly imperfect results have involved the expenditure of some little time and effort* and it was necessary to consider how best to utilise our limited resources.

Having chiefly before my eyes the needs of laboratory workers, I felt sure that the cases of m and n not greater than 25 were of the most importance. Probably in the type of problem alluded to on p. 73 the "control" should be regarded as our n and it is usually possible to arrange the experiments in such a fashion that animals at least equal in number to those specially tested serve as the control. When it is possible to plan a large control, it is usually practicable to fix the number arbitrarily, so that it seemed sufficient to give in tabular form the results of small samples after first samples of 50 and 100 without calculating the intermediate cases.

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* I desire heartily to thank my assistant, Mr J. W. Brown of the Lister Institute Statistical Department, to whose zealous co-operation in the arithmetical work I am greatly indebted.

The next question is as to whether, within the limited field chosen, interpolation can be trusted. Accurate methods of interpolation in the case of double-entry tables are a little complex* and not likely to appeal to the man in the laboratory. What is really material is whether simple interpolation is likely to lead to seriously erroneous conclusions.

I now proceed to some tests.

(1) A first sample of 17 having given 3 successes, required the probability that a second sample of 14 will contain 4 or more successes.

From the tables for $n = 20$, $p = 3$, $m = 10$ and $n = 15$, $p = 3$, $m = 10$, we have for the proportional frequency of 4 or more successes in m trials,

$$12{\cdot}8062$$
$$23{\cdot}0040$$
$$10{\cdot}1978$$

which gives by simple interpolation for $n = 17$, $p = 3$, $m = 10$,

$$18{\cdot}92488 \qquad (\alpha).$$

Similarly interpolating between the values for $n = 20$, $p = 3$, $m = 15$ and $n = 15$, $p = 3$, $m = 15$, we have for $n = 17$, $p = 3$, $m = 15$,

$$38{\cdot}92372 \qquad (\beta).$$

Interpolating between (α) and (β) we reach for the proportional frequency of 4 or more successes in 14 trials after $n = 17$, $p = 3$, 34·92.

The true value obtained by direct calculation is 35·07601, which gives an error of ·43 °/o in the interpolated value, a difference of no importance for such purposes as the present. In the accompanying table I have grouped together the results of a number of random trials made in different parts of the table. A perusal of these results leads, I think, to the following conclusions.

(1) For values of m and n ranging in each case up to 25, interpolation, when necessary, gives results of sufficient exactitude for all the purposes likely to be served by such tables.

(2) For greater values of m and n, particularly when the latter is greater than 50, the differences are too great to allow of interpolation and for such values the table can only provide the reader with a general impression (which is often enough sufficient) as to the limits within which possible variations from the proportions

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* *Vide* W. Palin Elderton, "Interpolation by Finite Differences (Two Independent Variables)," *Biometrika*, 1903, Vol. II. p. 105; W. Palin Elderton, "Some Notes on Interpolation in n-dimensional Space," *ibid.* 1908, Vol. VI. p. 94; also the Introduction to the British Association Tables of $F(r, \nu)$ and $H(r, \nu)$ Functions (issued by *B. A.* 1899, p. 56).

found in the first sample are likely to fall. As remarked above, it was thought that when the first sample exceeded fifty its numerical composition would often be at the choice of the worker. I think, therefore, that these tables are likely to serve most of the objects I had in mind when the work was undertaken, although it is much to be desired that someone will have leisure considerably to extend them. I do not see any immediate prospect of being that person.

The class of problem in which this species of investigation seems desirable has already been described and the reader is perhaps not anxious to see any more arithmetical examples. I may, however, give a single concrete instance of the kind of research in which, I hope, the tables will be of value.

Tests of the Accuracy of Simple Interpolation.

[The True Values are given in brackets.]

Example I. $n=22$, $p=4$, $m=16$.

0—3 Successes	1—3 Successes	3 Successes
57·38 (57·49)	51·27 (51·65)	18·70 (19·14)

Example II. $n=37$, $p=4$, $m=22$.

0—3 Successes	1—2 Successes	3 Successes
62·85 (67·89)	36·47 (39·83)	16·64 (18·88)

Example III. $n=71$, $p=4$, $m=43$.

0—2 Successes	1—3 Successes	3 Successes
46·43 (47·53)	50·60 (56·66)	15·21 (18·25)

Example IV. $n=100$, $p=4$, $m=39$.

0—3 Successes	1—2 Successes	3 Successes
83·84 (84·93)	48·59 (50·78)	13·78 (15·15)

In a paper by Rous*, several experiments of the following kind are detailed.

15 mice† were injected intraperitoneally with a suspension of mouse embryo in normal saline and 11 days later reinjected with the same substance. Ten days after the second injection they were inoculated subcutaneously with a mass made from mouse embryos 1·5 cm. long, and 17 previously untreated mice were inoculated at the same time to serve as a control.

In only one of the 17 control mice was no graft found at the autopsy, but 8 of the treated mice did not "take." If we wish to know whether this difference be an effect of the intraperitoneal inoculations, we may put $n=17$, $p=1$, and ascertain the chance that in $m=15$ there would be 8 or more successes.

From the tables, with interpolation, I find the odds against such a result to be about 260 to 1. In other words it is very likely that the treatment has led to the observed result. It may be remarked, however, that had we used a normal curve with S.D. $\sqrt{15 \cdot \frac{1 \times 16}{(17)^2}}$, the odds estimated therefrom would have been enormously greater.

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* "An Experimental Comparison of Transplanted Tumour and a Transplanted Normal Tissue Capable of Growth," by Peyton Rous, M.D., *Journ. Experimental Medicine*, 1910, Vol. XII. p. 344.

† I take the number stated in the text but can only identify 14 in the corresponding table.

In conclusion, I desire to refer to a subject indirectly related to the topic of this paper and, I think, of importance. We are familiar with such arrangements of material as the following. n_1 persons have been immunised against a certain disease and, having contracted the disease, a_1 have died. Of n_2 not immunised a_2 have died $\left(\frac{a_1}{n_1} < \frac{a_2}{n_2}\right)$. The extent of protection conferred is then estimated by some coefficient of correlation or association. The trustworthiness of the coefficient so calculated is then measured by a comparison between its arithmetical value and that of its standard deviation or "probable error." This process has its limitations. If, for instance, either a_1 or a_2 be zero, Yule's coefficients of association and colligation become unity and their standard deviations are indeterminate.

I would put forward for consideration the possibility that the use of Bayes' theorem might here be of value. Thus:

Let the chance of a_2 or more successes in n_2 after a_1 successes in n_1 be p_2 and the chance of a_1 or less successes in n_1 trials after a_2 successes in n_2 trials be p_1. Then, since either n_1 or n_2 might have been drawn first, a measure of the probability of the observed result will be $\frac{p_1 + p_2}{2}$.

We might indeed adopt a scale of reliability by putting $P = f\left(\frac{p_1 + p_2}{2}\right)$, the function being such that P increases to unity as $\frac{p_1 + p_2}{2}$ diminishes to zero.

I put forward these suggestions with some doubt, but I cannot help feeling sure that in such cases as those I have instanced the ordinary method of testing the reliability of a coefficient of association is a dangerous and possibly misleading artifice*.

In conclusion I desire to express my regret that this paper is so imperfect. The problems treated require mathematical abilities and training not at my command. I have only ventured to write upon the subject because of its practical importance and may, perhaps, venture to entertain the hope that my numerous mistakes of omission and commission will be leniently treated.

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* [The probability corresponding to the χ^2 of the fourfold table can be calculated straight away; but the difficulty arises from our not mentally appreciating grades of probability with the readiness we appreciate grades of correlation on a limited scale. EDITOR.]

TABLE. *Percentage Frequency of Successes in a Second Sample "m" after drawing "p" Successes in a First Sample "n".*

Successes		$p=0$	$p=1$	$p=2$	$p=3$	$p=4$	$p=5$
$n=6$	*0*	58·3333	31·8182	15·9091	7·0707		
$m=5$	*1*	26·5151	31·8182	26·5151	17·6768		
	2	10·6060	21·2121	26·5151	25·2525		
	3	3·5354	10·6060	18·9394	25·2525		
	4	·8838	3·7879	9·4697	17·6768		
	5	·1263	·7576	2·6515	7·0707		
$n=6$	*0*	53·8462	26·9231	12·2378	4·8951		
$m=6$	*1*	26·9231	29·3706	22·0280	13·0536		
	2	12·2378	22·0280	24·4755	20·3963		
	3	4·8951	13·0536	20·3962	23·3100		
	4	1·6317	6·1189	13·1119	20·3963		
	5	·4079	2·0979	6·1189	13·0536		
	6	·0582	·4079	1·6317	4·8951		
$n=7$	*0*	61·5385	35·8974	19·5804	9·7902		
$m=5$	*1*	25·6410	32·6340	29·3706	21·7560		
	2	9·3240	19·5804	26·1072	27·1950		
	3	2·7972	8·7024	16·3170	23·3100		
	4	·6216	2·7195	6·9930	13·5975		
	5	·0777	·4662	1·6317	4·3512		
$n=7$	*0*	57·1429	30·7692	15·3846	6·9930		
$m=6$	*1*	26·3736	30·7692	25·1748	16·7832		
	2	10·9890	20·9790	25·1748	23·3100		
	3	3·9960	11·1888	18·6480	23·3100		
	4	1·1988	4·6620	10·4895	17·4825		
	5	·2664	1·3986	4·1958	9·3240		
	6	·0333	·2331	·9324	2·7972		
$n=7$	*0*	53·3333	26·6667	12·3077	5·1282		
$m=7$	*1*	26·6667	28·7179	21·5385	13·0536		
	2	12·3077	21·5385	23·4965	19·5804		
	3	5·1282	13·0536	19·5804	21·7560		
	4	1·8648	6·5268	13·0536	19·0365		
	5	·5594	2·6107	6·8531	13·0536		
	6	·1243	·7615	2·6107	6·5268		
	7	·0155	·1243	·5594	1·8648		
$n=8$	*0*	64·2857	39·5604	23·0769	12·5874	6·2937	
$m=5$	*1*	24·7253	32·9670	31·4685	25·1748	17·4825	
	2	8·2418	17·9820	25·1748	27·9720	26·2238	
	3	2·2478	7·1928	13·9860	20·9790	26·2238	
	4	·4495	1·9980	5·2448	10·4895	17·4825	
	5	·0499	·2997	1·0489	2·7972	6·2937	
$n=8$	*0*	60·0000	34·2857	18·4615	9·2308	4·1958	
$m=6$	*1*	25·7143	31·6484	27·6923	20·1398	12·5874	
	2	9·8901	19·7802	25·1748	25·1748	20·9790	
	3	3·2967	9·5904	16·7832	22·3776	24·4755	
	4	·8991	3·5964	8·3916	14·6853	20·9790	
	5	·1798	·9590	2·9370	6·7133	12·5874	
	6	·0200	·1399	·5594	1·6783	4·1958	
$n=8$	*0*	56·2500	30·0000	15·0000	6·9231	2·8846	
$m=7$	*1*	26·2500	30·0000	24·2308	16·1538	9·1783	
	2	11·2500	20·7692	24·2308	22·0280	16·5210	
	3	4·3269	11·5385	18·3566	22·0280	21·4161	
	4	1·4423	5·2448	11·0140	17·1329	21·4161	
	5	·3934	1·8881	5·1399	10·2797	16·5210	
	6	·0787	·4895	1·7132	4·4056	9·1783	
	7	·0087	·0699	·3147	1·0489	2·8846	

TABLE—(*continued*).

Successes		$p=0$	$p=1$	$p=2$	$p=3$	$p=4$	$p=5$
$n=8$	*0*	52·9412	26·4706	12·3529	5·2941	2·0362	
$m=8$	*1*	26·4706	28·2353	21·1765	13·0317	6·7873	
	2	12·3529	21·1765	22·8054	19·0045	12·9576	
	3	5·2941	13·0317	19·0045	20·7322	18·1407	
	4	2·0362	6·7873	12·9576	18·1407	20·1563	
	5	·6787	2·9617	7·2563	12·9000	18·1407	
	6	·1851	1·0366	3·2250	7·2563	12·9576	
	7	·0370	·2633	1·0366	2·9617	6·7873	
	8	·0041	·0370	·1851	·6787	2·0362	
$n=9$	*0*	66·6667	42·8571	26·3736	15·3846	8·3916	
$m=5$	*1*	23·8095	32·9670	32·9670	27·9720	20·9790	
	2	7·3260	16·4835	23·9760	27·9720	27·9720	
	3	1·8315	5·9940	11·9880	18·6480	24·4755	
	4	·3330	1·4985	3·9960	8·1585	13·9860	
	5	·0333	·1998	·6993	1·8648	4·1958	
$n=9$	*0*	62·5000	37·5000	21·4286	11·5385	5·7692	
$m=6$	*1*	25·0000	32·1429	29·6703	23·0769	15·7343	
	2	8·9286	18·5439	24·7253	26·2238	23·6014	
	3	2·7472	8·2418	14·9850	20·9790	24·4755	
	4	·6868	2·8097	6·7433	12·2378	18·3566	
	5	·1249	·6743	2·0979	4·8951	9·4405	
	6	·0125	·0874	·3496	1·0489	2·6224	
$n=9$	*0*	58·8235	33·0882	17·6471	8·8235	4·0724	
$m=7$	*1*	25·7353	30·8824	26·4706	19·0045	11·8778	
	2	10·2941	19·8529	24·4344	23·7557	19·4364	
	3	3·6765	10·1810	16·9683	21·5961	22·6759	
	4	1·1312	4·2421	9·2554	15·1172	20·1563	
	5	·2828	1·3883	3·8873	8·0625	13·6055	
	6	·0514	·3239	1·1518	3·0234	6·4788	
	7	·0051	·0411	·1851	·6170	1·6968	
$n=9$	*0*	55·5555	29·4118	14·7059	6·8627	2·9412	
$m=8$	*1*	26·1438	29·4118	23·5294	15·6863	9·0498	
	2	11·4379	20·5882	23·5294	21·1161	15·8371	
	3	4·5752	11·7647	18·0995	21·1161	20·1563	
	4	1·6340	5·6561	11·3122	16·7969	20·1563	
	5	·5027	2·2624	5·7589	10·7500	16·1250	
	6	·1257	·7199	2·3036	5·3750	10·0782	
	7	·0229	·1645	·6582	1·9197	4·5249	
	8	·0023	·0206	·1028	·3771	1·1312	
$n=9$	*0*	52·6316	26·3158	12·3839	5·4180	2·1672	
$m=9$	*1*	26·3158	27·8638	20·8978	13·0031	6·9659	
	2	12·3839	20·8978	22·2910	18·5759	12·8602	
	3	5·4180	13·0031	18·5759	20·0047	17·5042	
	4	2·1672	6·9659	12·8602	17·5042	19·0955	
	5	·7740	3·2151	7·5018	12·7303	17·1859	
	6	·2381	1·2503	3·6372	7·6382	12·7303	
	7	·0595	·3897	1·4029	3·6372	7·5018	
	8	·0108	·0877	·3897	1·2503	3·2150	
	9	·0011	·0108	·0595	·2381	·7740	
$n=5$	*0*	54·5454	27·2727	12·1212	4·5454		
$m=5$	*1*	27·2727	30·3030	22·7273	12·9870		
	2	12·1212	22·7273	25·9740	21·6450		
	3	4·5454	12·9870	21·6450	25·9740		
	4	1·2987	5·4112	12·9870	22·7273		
	5	·2165	1·2987	4·5454	12·1212		

Percentage Frequencies of Successes in a Second Sample "m".

Successes		$p=0$	$p=1$	$p=2$	$p=3$	$p=4$	$p=5$	$p=6$	$p=7$
$n=10$ $m=5$	0	68·7500	45·8333	29·4643	18·1318	10·5769	5·7692		
	1	22·9167	32·7381	33·9972	30·2198	24·0385	17·3077		
	2	6·5476	15·1099	22·6648	27·4725	28·8461	26·9230		
	3	1·5110	5·0366	10·3022	16·4835	22·4359	26·9230		
	4	·2518	1·1447	3·0907	6·4103	11·2179	17·3077		
	5	·0229	·1373	·4807	1·2820	2·8846	5·7692		
$n=10$ $m=10$	0	52·3809	26·1905	12·4060	5·5138	2·2704	·8514		
	1	26·1905	27·5689	20·6767	12·9736	7·0949	3·4056		
	2	12·4060	20·6767	21·8930	18·2441	12·7709	7·6625		
	3	5·5138	12·9736	18·2441	19·4604	17·0278	12·5744		
	4	2·2704	7·0949	12·7709	17·0278	18·3377	16·5039		
	5	·8514	3·4056	7·6625	12·5744	16·5039	18·0043		
	6	·2838	1·4190	3·9295	7·8590	12·5030	16·5039		
	7	·0811	·4990	1·6841	4·0826	7·8590	12·5744		
	8	·0187	·1403	·5741	1·6841	3·9295	7·6625		
	9	·0031	·0284	·1403	·4990	1·4190	3·4056		
	10	·0003	·0031	·0187	·0811	·2838	·8514		
$n=15$ $m=5$	0	76·1905	57·1429	42·1053	30·4094	21·4654	14·7575	9·8383	6·3246
	1	19·0476	30·0752	35·0877	35·7757	33·5397	29·5149	24·5958	19·4604
	2	4·0100	10·0251	16·5119	22·3598	26·8318	29·5149	30·2717	29·1906
	3	·6683	2·3588	5·1599	8·9439	13·4159	18·1631	22·7038	26·5369
	4	·0786	·3685	1·0320	2·2360	4·1280	6·8111	10·3199	14·5953
	5	·0049	·0295	·1032	·2752	·6192	1·2384	2·2704	3·8921
$n=15$ $m=10$	0	61·5384	36·9231	21·5385	12·1739	6·6403	3·4783	1·7391	·8238
	1	24·6154	30·7692	28·0936	22·1344	15·8103	10·4348	6·4073	3·6613
	2	9·2308	18·0602	22·9857	23·7154	21·3439	17·2997	12·8146	8·7226
	3	3·2107	8·7565	14·5941	18·9723	20·9694	20·5034	18·0913	14·5376
	4	1·0216	3·6485	7·6619	12·2322	16·3095	18·9958	19·7873	18·6566
	5	·2919	1·3135	3·3874	6·5238	10·3613	14·2469	17·4128	19·1897
	6	·0730	·4032	1·2546	2·8781	5·3965	8·7064	12·4377	15·9914
	7	·0153	·1024	·3795	1·0279	2·2614	4·2644	7·1073	10·6609
	8	·0025	·0203	·0889	·2827	·7269	1·5991	3·1094	5·4516
	9	·0003	·0028	·0145	·0538	·1615	·4146	·9423	1·9383
	10	·0000	·0002	·0012	·0054	·0189	·0565	·1508	·3661
$n=15$ $m=15$	0	51·6129	25·8065	12·4583	5·7842	2·5707	1·0876	·4351	·1631
	1	25·8065	26·6963	20·0222	12·8538	7·4156	3·9155	1·9033	·8512
	2	12·4583	20·0222	20·7638	17·3032	12·4583	7·9941	4·6342	2·4375
	3	5·7842	12·8538	17·3032	17·9953	15·7459	12·0490	8·2152	5·0297
	4	2·5707	7·4156	12·4583	15·7459	16·4305	14·7874	11·7361	8·2991
	5	1·0876	3·9155	7·9941	12·0490	14·7874	15·4916	14·2006	11·5313
	6	·4351	1·9033	4·6342	8·2152	11·7361	14·2006	14·9480	13·8803
	7	·1631	·8512	2·4375	5·0297	8·2991	11·5313	13·8803	14·6968
	8	·0567	·3482	1·1607	2·7663	5·2415	8·3282	11·4309	13·7783
	9	·0181	·1289	·4965	1·3589	2·9443	5·3344	8·3350	11·4309
	10	·0052	·0426	·1882	·5889	1·4548	3·0006	5·3344	8·3282
	11	·0013	·0122	·0618	·2204	·6200	1·4548	2·9443	5·2415
	12	·0003	·0029	·0169	·0689	·2204	·5889	1·3589	2·7664
	13	·0001	·0006	·0037	·0170	·0618	·1882	·4965	1·1607
	14	·0000	·0001	·0006	·0029	·0122	·0426	·1290	·3482
	15	·0000	·0000	·0000	·0003	·0013	·0052	·0181	·0567

TABLE—(*continued*).

Successes		$p=0$	$p=1$	$p=2$	$p=3$	$p=4$	$p=5$
$n=20$, $m=5$	*0*	80·7692	64·6154	51·1538	40·0334	30·9349	23·5695
	1	16·1538	26·9231	33·3612	36·3940	36·8273	35·3542
	2	2·6923	7·0234	12·1313	17·3305	22·0964	26·0505
	3	·3512	1·2770	2·8884	5·1992	8·1408	11·5780
	4	·0319	·1520	·4333	·9577	1·8090	3·0647
	5	·0015	·0091	·0319	·0851	·1915	·3831
$n=20$, $m=10$	*0*	67·7419	45·1613	29·5884	19·0211	11·9763	7·3700
	1	22·5806	31·1457	31·7019	28·1795	23·0313	17·6880
	2	7·0078	15·0167	21·1346	24·3861	24·8738	23·2155
	3	2·0022	5·9325	10·8382	15·6071	19·3463	21·5333
	4	·5191	1·9965	4·5521	7·9661	11·7760	15·4158
	5	·1198	·5750	1·5932	3·3250	5·7809	8·8091
	6	·0240	·1398	·4618	1·1335	2·2940	4·0375
	7	·0040	·0278	·1079	·3084	·7210	1·4571
	8	·0005	·0043	·0193	·0636	·1707	·3946
	9	·0000	·0004	·0024	·0089	·0274	·0722
	10	·0000	·0000	·0002	·0006	·0023	·0068
$n=20$, $m=15$	*0*	58·3333	33·3333	18·6275	10·1604	5·3977	2·7859
	1	25·0000	29·4118	25·4011	19·0508	13·0590	8·3578
	2	10·2941	18·7166	22·2259	21·5090	18·2826	14·1218
	3	4·0553	10·1381	15·5343	18·6411	19·1232	17·4841
	4	1·5207	4·9056	9·3206	13·4987	16·3913	17·4841
	5	·5396	2·1584	4·9495	8·4849	12·0203	14·7942
	6	·1799	·8683	2·3569	4·7138	7·7053	10·8491
	7	·0558	·3190	1·0101	2·3310	4·3590	6·9744
	8	·0159	·1063	·3885	1·0256	2·1795	3·9421
	9	·0041	·0318	·1330	·3989	·9581	1·9511
	10	·0010	·0084	·0399	·1353	·3658	·8362
	11	·0002	·0019	·0102	·0391	·1188	·3041
	12	·0000	·0004	·0022	·0093	·0317	·0907
	13	·0000	·0001	·0004	·0017	·0065	·0209
	14	·0000	·0000	·0000	·0002	·0009	·0033
	15	·0000	·0000	·0000	·0000	·0001	·0003
$n=20$, $m=20$	*0*	51·2195	25·6098	12·4765	5·9099	2·7154	1·2068
	1	25·6098	26·2664	19·6998	12·7783	7·5427	4·1377
	2	12·4765	19·6998	20·2323	16·8602	12·2839	8·0929
	3	5·9099	12·7783	16·8602	17·3419	15·1742	11·7715
	4	2·7154	7·5427	12·2839	15·1742	15·6340	14·0706
	5	1·2068	4·1377	8·0929	11·7715	14·0706	14·5245
	6	·5172	2·1297	4·9048	8·2768	11·3473	13·3141
	7	·0839	1·0326	2·7589	5·3399	8·3213	11·0186
	8	·0315	·4719	1·4462	3·1817	5·5954	8·3131
	9	·0112	·2030	·7070	1·7554	3·4638	5·7473
	10	·0037	·0819	·3218	·8965	1·9757	3·6474
	11	·0012	·0308	·1358	·4226	1·0362	2·1221
	12	·0003	·0107	·0528	·1829	·4974	1·1274
	13	·0001	·0034	·0188	·0720	·2168	·5429
	14	·0000	·0010	·0060	·0255	·0848	·2345
	15	—	·0003	·0017	·0080	·0293	·0893
	16	—	·0000	·0004	·0022	·0087	·0293
	17	—	—	·0001	·0005	·0022	·0080
	18	—	—	·0000	·0001	·0004	·0017
	19	—	—	—	·0000	·0001	·0003
	20	—	—	—	—	·0000	·0000

TABLE—(*continued*).

Successes		$p=0$	$p=1$	$p=2$	$p=3$	$p=4$	$p=5$
$n=25$	0	83·8710	69·8925	57·8421	47·5131	38·7144	31·2693
$m=5$	1	13·9785	24·1008	30·9868	35·1949	37·2254	37·5232
	2	1·9281	5·1645	9·1813	13·5365	17·8682	21·8885
	3	·2065	·7651	1·7656	3·2488	5·2115	7·6134
	4	·0153	·0736	·2119	·4738	·9064	1·5573
	5	·0006	·0035	·0123	·0329	·0741	·1483
$n=25$	0	72·2222	51·5873	36·4146	25·3798	17·4486	11·8200
$m=10$	1	20·6349	30·3455	33·1041	31·7248	28·1430	23·6401
	2	5·4622	12·4141	18·6211	23·0261	25·3287	25·6780
	3	1·3242	4·1380	8·0091	12·2806	16·3035	19·5642
	4	·2897	1·1680	2·8032	5·1875	8·1518	11·4125
	5	·0561	·2803	·8119	1·7786	3·2607	5·2673
	6	·0093	·0564	·1933	·4940	1·0451	1·9313
	7	·0013	·0092	·0368	·1086	·2628	·5518
	8	·0001	·0011	·0053	·0179	·0493	·1170
	9	·0000	·0001	·0005	·0020	·0062	·0165
	10	·0000	·0000	·0000	·0001	·0004	·0012
$n=25$	0	63·4146	39·6341	24·3902	14·7625	8·7777	5·1203
$m=15$	1	23·7805	30·4878	28·8832	23·9392	18·2869	13·1666
	2	8·5366	16·8485	21·8575	23·2742	21·9443	18·9753
	3	2·9204	7·8930	13·1550	17·2894	19·5777	19·9337
	4	·9472	3·2888	6·7654	10·6788	14·2383	16·8191
	5	·2894	1·2403	3·0643	5·6953	8·8100	11·9361
	6	·0827	·4256	1·2381	2·6697	4·7366	7·2943
	7	·0218	·1326	·4477	1·1072	2·2329	3·8807
	8	·0053	·0373	·1444	·4060	·9240	1·8017
	9	·0012	·0094	·0412	·1307	·3337	·7266
	10	·0002	·0020	·0102	·0364	·1038	·2515
	11	·0000	·0004	·0022	·0086	·0272	·0732
	12	·0000	·0001	·0004	·0016	·0058	·0173
	13	·0000	·0000	·0001	·0002	·0009	·0031
	14	—	·0000	·0000	·0000	·0001	·0004
	15	—	—	·0000	·0000	·0000	·0000
$n=25$	0	56·5217	31·4010	17·1278	9·1614	4·7988	2·4579
$m=20$	1	25·1208	28·5463	23·8993	17·4502	11·7044	7·3738
	2	10·8476	18·9202	21·6231	20·2168	16·6788	12·5733
	3	4·5409	10·8116	15·8218	18·1951	17·9618	15·8820
	4	1·8380	5·6036	10·0864	13·8796	16·0711	16·4186
	5	·7172	2·6897	5·7932	9·3504	12·5094	14·5943
	6	·2690	1·2069	3·0491	5·6861	8·6871	11·4669
	7	·0965	·5082	1·4833	3·1589	5·4604	8·0943
	8	·0330	·2009	·6695	1·6133	3·1317	5·1816
	9	·0107	·0744	·2806	·7592	1·6449	3·0226
	10	·0033	·0257	·1089	·3290	·7916	1·6088
	11	·0009	·0082	·0390	·1308	·3482	·7800
	12	·0002	·0024	·0128	·0475	·1393	·3429
	13	·0001	·0006	·0038	·0156	·0502	·1357
	14	·0000	·0002	·0010	·0046	·0162	·0477
	15	—	·0000	·0002	·0012	·0045	·0147
	16	—	—	·0001	·0002	·0011	·0039
	17	—	—	—	·0000	·0002	·0008
	18	—	—	—	—	·0000	·0001
	19	—	—	—	—	—	·0000
	20	—	—	—	—	—	—

TABLE—(*continued*).

Successes		$p=0$	$p=1$	$p=2$	$p=3$	$p=4$	$p=5$
$n=25$	*0*	50·9804	25·4902	12·4850	5·9824	2·8003	1·2784
$m=25$	*1*	25·4902	26·0104	19·5078	12·7285	7·6094	4·2613
	2	12·4850	19·5078	19·9229	16·6024	12·1751	8·1352
	3	5·9824	12·7285	16·6024	16·9713	14·8499	11·6037
	4	2·8003	7·6094	12·1751	14·8499	15·1953	13·6757
	5	1·2784	4·2613	8·1352	11·6037	13·6757	14·0093
	6	·5682	2·2598	5·0451	8·2883	11·1185	12·8418
	7	·2453	1·1411	2·9344	5·4870	8·2990	10·7251
	8	·1027	·5502	1·6103	3·3951	5·7456	8·2555
	9	·0416	·2535	·8365	1·9732	3·7128	5·9003
	10	·0162	·1115	·4118	1·0801	2·2477	3·9335
	11	·0061	·0468	·1921	·5573	1·2771	2·4521
	12	·0022	·0187	·0848	·2709	·6811	1·4304
	13	·0007	·0070	·0353	·1238	·3406	·7802
	14	·0002	·0025	·0138	·0531	·1592	·3971
	15	—	·0008	·0051	·0212	·0693	·1879
	16	—	·0003	·0017	·0079	·0280	·0822
	17	—	·0001	·0005	·0027	·0104	·0330
	18	—	—	·0002	·0008	·0035	·0120
	19	—	—	·0000	·0002	·0010	·0039
	20	—	—	—	·0001	·0003	·0011
	21	—	—	—	—	·0001	·0003
	22	—	—	—	—	—	·0001
	23	—	—	—	—	—	—
	24	—	—	—	—	—	—
	25	—	—	—	—	—	—
$n=50$	*0*	91·0714	82·7922	75·1263	68·0389	61·4967	55·4676
$m=5$	*1*	8·2792	15·3319	21·2621	26·1688	30·1454	33·2805
	2	·6133	1·7357	3·2711	5·1311	7·2349	9·5087
	3	·0347	·1335	·3207	·6157	1·0335	1·5848
	4	·0013	·0065	·0192	·0440	·0861	·1517
	5	·0000	·0001	·0005	·0014	·0033	·0066
$n=50$	*0*	83·6065	69·6721	57·8633	47·8869	39·4857	32·4346
$m=10$	*1*	13·9344	23·6177	29·9293	33·6048	35·2551	35·3833
	2	2·1256	5·4972	9·4513	13·5019	17·3070	20·6402
	3	·2932	1·0287	2·2503	3·9278	5·9827	8·3080
	4	·0360	·1607	·4296	·8910	1·5803	2·5164
	5	·0039	·0210	·0668	·1614	·3282	·5921
	6	·0003	·0023	·0084	·0233	·0536	·1085
	7	·0000	·0002	·0008	·0026	·0067	·0152
	8	·0000	·0000	·0001	·0002	·0006	·0015
	9	·0000	·0000	·0000	·0000	·0000	·0001
	10	·0000	·0000	·0000	·0000	·0000	·0000
$n=50$	*0*	77·2727	59·4406	45·5092	34·6737	26·2849	19·8214
$m=15$	*1*	17·8322	27·8628	32·5066	33·5552	32·3175	29·7321
	2	3·9008	9·2876	14·6804	19·2530	22·6222	24·6927
	3	·8049	2·5965	5·2143	8·3429	11·6306	14·7589
	4	·1558	·6385	1·5643	2·9695	4·8127	6·9910
	5	·0281	·1405	·4083	·9011	1·6718	2·7465
	6	·0047	·0278	·0939	·2371	·4976	·9155
	7	·0007	·0049	·0191	·0544	·1279	·2616
	8	·0001	·0008	·0034	·0109	·0284	·0642
	9	—	·0001	·0005	·0019	·0054	·0132
	10	—	—	·0001	·0003	·0009	·0024
	11	—	—	—	—	·0001	·0003
	12	—	—	—	—	—	·0000
	13, 14, 15	—	—	—	—	—	—

TABLE—(*continued*).

Successes		$p=0$	$p=1$	$p=2$	$p=3$	$p=4$	$p=5$
$n=50$	*0*	71·8310	51·3078	36·4360	25·7195	18·0421	12·5748
$m=20$	*1*	20·5231	29·7437	32·1494	30·7099	27·3365	23·2150
	2	5·6513	12·4661	18·2340	22·1018	23·9720	24·1218
	3	1·4959	4·4655	8·2882	12·2410	15·7316	18·3785
	4	·3796	1·4377	3·2515	5·6902	8·4901	11·3384
	5	·0920	·4247	1·1380	2·3122	3·9438	5·9480
	6	·0212	·1161	·3613	·8391	1·6163	2·7262
	7	·0046	·0295	·1049	·2751	·5926	1·1089
	8	·0010	·0070	·0279	·0820	·1959	·4039
	9	·0002	·0015	·0068	·0222	·0585	·1323
	10	·0000	·0003	·0015	·0055	·0158	·0390
	11	—	·0001	·0003	·0012	·0039	·0103
	12	—	—	·0001	·0002	·0008	·0024
	13	—	—	—	·0000	·0002	·0005
	14	—	—	—	—	·0000	·0001
	15—20	—	—	—	—	—	·0000
$n=50$	*0*	67·1053	44·7368	29·6230	19·4782	12·7149	8·2378
$m=25$	*1*	22·3684	30·2276	30·4346	27·0530	22·3854	17·6525
	2	7·2546	14·9068	20·2898	22·8617	23·0250	21·4900
	3	2·2857	6·3492	10·9546	15·0234	17·9083	19·3831
	4	·6984	2·4592	5·1643	8·3826	11·5877	14·3204
	5	·2066	·8853	2·2004	4·1420	6·5376	9·1130
	6	·0590	·2994	·8629	1·8546	3·3018	5·1406
	7	·0163	·0956	·3146	·7627	1·5167	2·6162
	8	·0043	·0289	·1073	·2904	·6398	1·2147
	9	·0011	·0083	·0343	·1029	·2494	·5181
	10	·0003	·0022	·0103	·0340	·0901	·2038
	11	·0001	·0006	·0029	·0105	·0302	·0741
	12	·0000	·0001	·0008	·0030	·0094	·0249
	13	·0000	·0000	·0002	·0008	·0027	·0077
	14	—	·0000	·0000	·0002	·0007	·0022
	15	—	—	·0000	·0000	·0002	·0006
	16	—	—	—	·0000	·0000	·0001
	17	—	—	—	—	·0000	·0000
	18—25	—	—	—	—	—	·0000
$n=50$	*0*	50·4950	25·2475	12·4963	6·1206	2·9657	1·4210
$m=50$	*1*	25·2475	25·5026	19·1269	12·6198	7·7231	4·4875
	2	12·4963	19·1269	19·3241	16·1034	11·9504	8·1873
	3	6·1206	12·6198	16·1034	16·2729	14·2388	11·2686
	4	2·9657	7·7231	11·9504	14·2388	14·3919	12·9527
	5	1·4210	4·4875	8·1873	11·2686	12·9527	13·0951
	6	·6731	2·5063	5·2821	8·2677	10·6753	12·0038
	7	·3151	1·3552	3·2480	5·7108	8·2014	10·1734
	8	·1457	·7126	1·9185	3·7517	5·9437	8·0780
	9	·0665	·3654	1·0942	2·3606	4·0975	6·0662
	10	·0300	·1831	·6049	1·4298	2·7034	4·3381
	11	·0133	·0898	·3250	·8367	1·7147	2·9694
	12	·0058	·0431	·1699	·4743	1·0490	1·9531
	13	·0025	·0203	·0866	·2610	·6205	1·2381
	14	·0011	·0093	·0431	·1396	·3557	·7582
	15	·0004	·0042	·0209	·0726	·1978	·4493
	16	·0002	·0019	·0099	·0368	·1068	·2580
	17	·0001	·0008	·0046	·0182	·0561	·1437
	18	—	·0003	·0021	·0088	·0286	·0777
	19	—	·0001	·0009	·0041	·0142	·0408
	20	—	—	·0004	·0019	·0069	·0208
	21	—	—	·0002	·0008	·0032	·0103
	22	—	—	·0001	·0004	·0015	·0050
	23	—	—	—	·0002	·0007	·0023
	24	—	—	—	·0001	·0003	·0010
	25	—	—	—	·0000	·0001	·0005
	26	—	—	—	—	·0000	·0002
	27	—	—	—	—	·0000	·0001
	28—50	—	—	—	—	—	·0000

TABLE—(*continued*).

	sses	$p=0$	$p=1$	$p=2$	$p=3$	$p=4$	$p=5$	$p=6$	$p=7$	$p=8$	$p=9$
0	*0*	90·9910	82·7191	75·1302	68·1737	61·8023	55·9719	50·6412	45·7719	41·3280	37·2763
.0	*1*	8·2719	15·1778	20·8695	25·4855	29·1520	31·9839	34·0855	35·5510	36·4659	36·9072
	2	·6830	1·8972	3·5107	5·4097	7·4962	9·6874	11·9134	14·1158	16·2472	18·2690
	3	·0506	·1891	·4416	·8243	1·3455	2·0065	2·8031	3·7269	4·7658	5·9052
	4	·0033	·0156	·0442	·0971	·1829	·3098	·4857	·7174	1·0109	1·3708
	5	·0002	·0011	·0036	·0090	·0194	·0368	·0641	·1044	·1609	·2374
	6	·0000	·0001	·0002	·0007	·0016	·0034	·0065	·0115	·0194	·0309
	7	—	·0000	·0000	·0000	·0001	·0002	·0005	·0010	·0017	·0030
	8	—	—	—	—	·0000	·0000	·0000	·0001	·0001	·0002
	9	—	—	—	—	—	—	—	·0000	·0000	·0000
	10	—	—	—	—	—	—	—	—	—	—

	$p=10$	$p=15$	$p=20$	$p=25$	$p=30$	$p=35$	$p=40$	$p=45$	$p=50$
0	33·5855	19·6056	11·0992	6·0712	3·1945	1·6083	·7697	·3473	·1463
1	36·9441	33·0200	25·8982	18·5708	12·3788	7·7198	4·5082	2·4580	1·2434
2	20·1513	26·8727	28·8081	26·8613	22·5639	17·3696	12·3485	8·1229	4·9313
3	7·1284	13·8698	20·0784	24·1644	25·4567	24·1112	20·8229	16·5036	12·0167
4	1·8005	5·0127	9·6930	14·9554	19·6711	22·8554	23·9308	22·8255	19·9224
5	·3376	1·3220	3·3813	6·6468	10·8709	15·4516	19·5797	22·4513	23·4799
6	·0474	·2571	·8619	2·1464	4·3483	7·5418	11·5470	15·9030	19·9224
7	·0049	·0363	·1583	·4968	1·2424	2·6232	4·8456	8·0093	12·0167
8	·0003	·0036	·0200	·0788	·2425	·6221	1·3845	2·7446	4·9313
9	·0000	·0002	·0015	·0077	·0292	·0908	·2432	·5778	1·2434
10	—	·0000	·0001	·0004	·0017	·0062	·0199	·0567	·1463

	sses	$p=0$	$p=1$	$p=2$	$p=3$	$p=4$	$p=5$	$p=6$	$p=7$	$p=8$	$p=9$	$p=10$
0	*0*	95·2830	90·7457	86·3830	82·1896	78·1607	74·2914	70·5768	67·0123	63·5933	60·3153	57·1739
5	*1*	4·5373	8·7256	12·5800	16·1156	19·3467	22·2874	24·9514	27·3520	29·5020	31·4142	33·1007
	2	·1745	·5083	·9867	1·5956	2·3216	3·1517	4·0737	5·0756	6·1463	7·2749	8·4512
	3	·0051	·0199	·0488	·0957	·1642	·2573	·3780	·5287	·7117	·9287	1·1813
	4	·0001	·0005	·0015	·0034	·0067	·0119	·0197	·0306	·0454	·0649	·0899
	5	·0000	·0000	·0000	·0001	·0001	·0003	·0004	·0008	·0013	·0020	·0030
0	*0*	87·0690	75·7121	65·7500	57·0221	49·3852	42·7116	36·8873	31·8110	27·3928	23·5527	20·2198
15	*1*	11·3568	19·9243	26·1836	30·5476	33·3684	34·9458	35·5336	35·3456	34·5611	33·3293	31·7739
	2	1·3947	3·7027	6·5459	9·6321	12·7407	15·7096	18·4248	20·8110	22·8233	24·4415	25·6636
	3	·1604	·5730	1·2777	2·2767	3·5456	5·0426	6·7156	8·5076	10·3611	12·2207	14·0361
	4	·0172	·0774	·2091	·4386	·7879	1·2724	1·9006	2·6738	3·5865	4·6273	5·7796
	5	·0017	·0093	·0295	·0715	·1458	·2641	·4381	·6787	·9959	1·3973	1·8884
	6	·0002	·0010	·0037	·0100	·0229	·0461	·0842	·1428	·2278	·3459	·5036
	7	·0000	·0001	·0004	·0012	·0031	·0068	·0137	·0252	·0435	·0711	·1112
	8	—	·0000	·0000	·0001	·0004	·0009	·0019	·0037	·0070	·0122	·0204
	9	—	—	—	·0000	·0000	·0001	·0002	·0005	·0009	·0017	·0031
	10	—	—	—	—	—	·0000	·0000	·0001	·0001	·0002	·0004
	11—15	—	—	—	—	—	—	—	·0000	·0000	·0000	·0000
0	*0*	83·4711	69·5592	57·8686	48·0604	39·8449	32·9751	27·2403	22·4613	18·4859	15·1848	12·4488
20	*1*	13·9119	23·3813	29·4247	32·8618	34·3491	34·4088	33·4530	31·8036	29·7094	27·3600	24·8976
	2	2·2212	5·6472	9·5567	13·4563	17·0252	20·0718	22·4994	24·2787	25·4270	25·9920	26·0397
	3	·3388	1·1584	2·4716	4·2124	6·2724	8·5261	10·8479	13·1236	15·2562	17·1690	18·8065
	4	·0492	·2122	·5480	1·0993	1·8873	2·9118	4·1535	5·5775	7·1382	8·7832	10·4578
	5	·0068	·0354	·1077	·2490	·4853	·8394	1·3291	1·9649	2·7495	3·6775	4·7356
	6	·0009	·0054	·0191	·0500	·1093	·2099	·3658	·5913	·8994	1·3010	1·8040
	7	·0001	·0008	·0031	·0090	·0219	·0462	·0881	·1547	·2545	·3965	·5898
	8	·0000	·0001	·0004	·0015	·0039	·0090	·0187	·0356	·0630	·1053	·1675
	9	—	·0000	·0001	·0002	·0006	·0016	·0035	·0072	·0137	·0245	·0416
	10	—	—	·0000	·0000	·0001	·0002	·0006	·0013	·0026	·0050	·0091
	11	—	—	—	—	·0000	·0000	·0001	·0002	·0005	·0009	·0017
	12	—	—	—	—	—	—	·0000	·0000	·0001	·0001	·0003
	13—20	—	—	—	—	—	—	—	—	·0000	·0000	·0000

TABLE—(*continued*).

Successes		$p=0$	$p=1$	$p=2$	$p=3$	$p=4$	$p=5$	$p=6$	$p=7$	$p=8$	$p=9$	$p=10$
$n=100$	*0*	80·1587	64·1270	51·1981	40·7920	32·4330	25·7320	20·3711	16·0915	12·6823	9·9724	7·823
$m=25$	*1*	16·0317	25·8576	31·2184	33·4361	33·5052	32·1649	29·9575	27·2737	24·3890	21·4922	18·707
	2	3·1029	7·5681	12·2826	16·5799	20·1031	22·7047	24·3723	25·1757	25·2300	24·6693	23·630
	3	·5802	1·9024	3·8912	6·3556	9·0661	11·8013	14·3734	16·6391	18·5020	19·9086	20·842
	4	·1046	·4323	1·0701	2·0562	3·3806	4·9929	6·8150	8·7536	10·7117	12·5970	14·329
	5	·0182	·0908	·2644	·5855	1·0922	1·8077	2·7378	3·8700	5·1757	6·6134	8·132
	6	·0030	·0178	·0597	·1501	·3138	·5764	·9606	1·4841	2·1565	2·9790	3·943
	7	·0005	·0033	·0125	·0351	·0815	·1647	·3000	·5035	·7910	1·1761	1·669
	8	·0001	·0005	·0024	·0075	·0193	·0426	·0844	·1531	·2589	·4127	·626
	9	·0000	·0001	·0004	·0015	·0042	·0100	·0215	·0421	·0763	·1299	·209
	10	—	·0000	·0001	·0003	·0008	·0022	·0050	·0105	·0203	·0369	·063
	11	—	—	·0000	·0001	·0001	·0004	·0011	·0024	·0049	·0095	·017
	12	—	—	—	·0000	·0000	·0001	·0002	·0005	·0011	·0022	·004
	13	—	—	—	—	—	·0000	·0000	·0001	·0002	·0005	·000
	14	—	—	—	—	—	—	—	·0000	·0000	·0001	·000
	15—25	—	—	—	—	—	—	—	—	—	·0000	·000

Successes		$p=0$	$p=1$	$p=2$	$p=3$	$p=4$	$p=5$	$p=6$	$p=7$	$p=8$	$p=9$	$p=10$
$n=100$	*0*	66·8874	44·5916	29·6280	19·6185	12·9456	8·5121	5·5769	3·6405	2·3676	1·5339	·99
$m=50$	*1*	22·2958	29·9273	30·0284	26·6919	22·1671	17·6113	13·5550	10·1832	7·5029	5·4395	3·88
	2	7·3322	14·8625	20·0189	22·3956	22·4728	20·9746	18·5789	15·8126	13·0370	10·4710	8·22
	3	2·3780	6·4708	10·9693	14·8274	17·4789	18·7745	18·8406	17·9434	16·3894	14·4635	12·39
	4	·7603	2·6038	5·3333	8·4691	11·4896	13·9817	15·7005	16·5656	16·6252	16·0095	14·887
	5	·2396	·9912	2·3852	4·3589	6·6996	9·1228	11·3492	13·1572	14·4085	15·0512	15·10
	6	·0743	·3614	1·0008	2·0720	3·5636	5·3759	7·3484	9·2958	11·0430	12·4505	13·42
	7	·0227	·1271	·3987	·9237	1·7600	2·9173	4·3512	5·9710	7·6559	9·2753	10·70
	8	·0068	·0433	·1520	·3901	·8167	1·4771	2·3900	3·5398	4·8771	6·3248	7·78
	9	·0020	·0143	·0557	·1572	·3590	·7044	1·2301	1·9578	2·8874	3·9946	5·23
	10	·0006	·0046	·0197	·0607	·1504	·3185	·5978	1·0184	1·6022	2·3574	3·27
	11	·0002	·0014	·0068	·0226	·0603	·1373	·2758	·5012	·8386	1·3088	1·92
	12	—	·0004	·0022	·0081	·0232	·0566	·1213	·2345	·4161	·6871	1·06
	13	—	·0001	·0007	·0028	·0086	·0224	·0510	·1046	·1965	·3425	·56
	14	—	—	·0002	·0009	·0031	·0085	·0206	·0447	·0886	·1627	·27
	15	—	—	·0001	·0003	·0011	·0031	·0080	·0183	·0382	·0738	·13
	16	—	—	—	·0001	·0004	·0011	·0030	·0072	·0158	·0320	·06
	17	—	—	—	—	·0001	·0004	·0011	·0027	·0063	·0133	·02
	18	—		—	—	—	·0001	·0004	·0010	·0024	·0053	·01
	19	—	—	—	—	—	—	·0001	·0003	·0009	·0020	·00
	20	—	—	—	—	—	—	—	·0001	·0003	·0008	·00
	21	—	—	—	—	—	—	—	—	·0001	·0003	·00
	22	—	—	—	—	—	—	—	—	—	·0001	·00
	23	—	—	—	—	—		—	—	—	—	·00
	24—50	—	—	—	—	—		—	—	—	—	—

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VACCINES AND FEVER.

BY

E. C. HORT.*

(From the Lister Institute: The Constance Trotter Researches.)

In the great majority of infective diseases accompanied by marked systemic disturbance the most prominent clinical feature is that form of intoxication which is expressed in fever. The relationship of fever to general infection is so constant that whenever continued fever occurs we may be almost certain that an infective process is at work. Continued fever is in fact an index both of the continued activity of micro organisms pathogenic to the individual infected and of the absence of effective protection against such activity. On the other hand, the absence of fever does not necessarily indicate the absence of infection. On the contrary, it is a familiar experience that in severe infections leading to a fatal issue there may be no fever on account of grave impairment of the reactive functions of the infected body. This impairment may exist prior to infection or may be the expression of extreme intoxication after infection has occurred. This observation can be verified with great ease. We have therefore learnt to recognize that whilst the continuance of fever is an excellent indicator of the continuance of infection and of the absence of effective immunity thereto, it is also an index that the power of reaction—whatever that may prove exactly to mean—is not yet lost. Now the power of reaction to infection in terms of fever is in some unknown way intimately associated with the complex problems presented by immunity. And we have some reason to believe that the mere presence of continued fever is a sure sign that the machinery of immunization has been set in motion and is still running. The solution therefore of the mystery that surrounds the nature and source of the substances that are the immediate cause of the fever of infective disease can hardly fail to throw light on many of those problems in immunity production which are at present totally obscure.

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* The protocols for many of the experiments referred to in this paper have already been published. In most of the experiments described in such protocols I was aided by Dr. W. J. Penfold. Full details of the additional experiments referred to in this paper will be published elsewhere, and for these I accept sole responsibility.

Hence determination of the essential cause of the fever of infective disease—from the pathological standpoint as opposed to that of physiology—is a matter of immediate concern to all students of immunity. And, as I shall show, this particularly applies to those who administer dead bacterial vaccines, whether for purposes of prophylaxis or of treatment, as well as to those who make use of bacterial emulsions in test tube reactions.

In all immunity problems, whether of the test tube or the living body, the keystone on which all inquiry rests has long been recognized to be the antigen, or substance that provokes the manifestation of antibodies. Similarly in the pathological problems presented by fever inquiry must always start from the pyrogen, or substance that provokes the manifestation of fever.

The antigens, then, and pyrogens of infective disease are substances so vital to the production of immunity and of fever that precise knowledge of their true source, as well as of their chemico-physical nature, must always be a necessary preliminary to an intelligible conception of how they act.

It is widely believed, largely in consequence of Pfeiffer's early work, that the main source of supply of both antigen and pyrogen in infective disease is dissolved bacterial protoplasm. In an infected subject the primary source of supply of these substances is therefore, we are taught, one foreign to that subject, whether the agents affecting their liberation be of an autolytic or heterolytic character. Moreover, since antigen and pyrogen are supposed to be genuine derivatives of bacterial protoplasm in solution, the theory lays down that it is only dead organisms that can supply them. The living bacterium as such has, we must infer—if we accept the theory—no antigenic or pyrogenetic values. Its potential worth, in terms of the production of antibodies and of fever, only becomes kinetic when bacterial death followed by bacterial solution and absorption has occurred.

So far as antigen is concerned, the theory that it is derived from dead bacterial protoplasm has in the main been accepted without question for many years. And incidentally it is the bedrock of that form of vaccine-therapy—the use of dead vaccines—which is under trial in this country to-day. So far as fever is concerned, the theory that the pyrogen of infective disease is a derivative of dead bacterial protoplasm in solution has also, as a rule, been accepted without serious question till comparatively recent date.[1]

In consequence of the general acceptance of this hypothesis as to the source of antigen and pyrogen in infective disease it might reasonably be supposed that it rests on a sound experimental basis. Whether this supposition be well grounded in the case of antigen is a question that cannot be discussed here. In the case, however, of pyrogen I shall show that the evidence hitherto relied on in favour of the view that it is a genuine derivative of dead bacterial protoplasm can no longer be accepted. On the contrary, I shall cite experimental data which suggest—if they do not conclusively prove—that the fever of infective disease is the direct result of absorption of pyrogen derived from the medium offered by the tissues of the infected host in consequence of the activity of the living organism or its products on such medium. And as a corollary I shall indicate the practical bearing of this displacement of current theory on the administration of dead vaccines for prophylactic or therapeutic purposes, as well as on all such test-tube experiments as involve the use of bacterial emulsions of the same nature.

The experimental basis of the theory that dead bacterial protoplasm is the direct source of the fever of infective disease is the undoubted fact that fever does frequently follow the injection into healthy subjects of dead cultures of certain organisms. At first sight this evidence would seem to be conclusive, especially if fever were found to follow the injection of all known forms of dead pathogenic bacteria and protozoa. It has, however, for some years been recognized that there are exceptions to what is by many still believed to be a general rule. For example, injection of dead cultures of the bacillus of Koch, *B. anthracis* and *B. diphtheriae* into healthy subjects has from time to time been found not to be followed by fever. In the case of the bacillus of Koch it was believed that the "waxy envelope" prevented bacterial solution and consequent extraction of the pyrogen with which this organism was credited. In the case of *B. anthracis* and *B. diphtheriae*, however, no explanation was forthcoming as to the absence of fever after injection of the killed organism. Nevertheless, these exceptions were looked upon as unimportant, and were to a great extent forgotten. When the practice of administering dead bacterial vaccines became general it was soon found that their injection might give rise to mild forms of intoxication, including fever. Prophylactic injections into healthy subjects of dead vaccines of *B. typhosus* are a case in point. Belief in dead bacteria as the primary source of pyrogen became therefore more firmly rooted than ever, though again no explanation was given—beyond a quantitative one—of the frequent experience that fever did not follow, for example, injections of certain staphylococcal vaccines. The subject, in fact, does not seem to have been investigated.

Early in 1911, therefore, I examined, with the aid of my laboratory colleague, Dr. Penfold, a large number of dead and living bacterial vaccines by injecting them, for the most part intravenously, into healthy rabbits. The organisms examined in respect of their pyrogenetic properties are given in the subjoined list. They are divided into two groups, according to whether they did or did not on intravenous injection produce immediate fever if the organisms were first killed. The character of the list given is, I think, sufficient answer to the very reasonable suggestion that the absence of immediate fever after injection of an emulsion of organisms might be due to difficulty of lysis of the particular organism. In all cases the vaccines injected were made from laboratory cultures. Many different strains of several of the organisms were examined, an important point, as will be seen, in the study of the relative pyrogenetic properties of any bacterial emulsion. Attention is drawn to the fact that the list of the organisms from laboratory cultures which did produce fever is a list also of Gram-negative organisms. On the other hand, the list of organisms injection of which did not produce fever is a list also of Gram-positive organisms. The parallelism is a striking one, though its interpretation is at present obscure.

PYROGENETIC VACCINES.

B. typhosus.
B. coli.
B. influenzae.
B. pestis.
B. melitensis.
M. catarrhalis.
V. cholerae.
Acne bacillus.
S. albus (certain strains).
S. citreus (certain strains).
S. aureus (certain strains).

APYROGENETIC VACCINES.

B. diphtheriae.
B. anthracis
Koch's bacillus.
B. mallei.
Streptococcus.
Pneumococcus.
Staphylococcus aureus (certain strains).
(Tuberculin.)
(Mallein.)

Clearly, then, the list of organisms that apparently do not contain any pyrogenetic substances is much longer than has previously been realized. And no doubt further examination will reveal yet other laboratory-grown organisms injection of which does not produce fever if they have been first killed. So far I have not examined for the presence or absence of pyrogenetic function such protozoa as can be grown on laboratory media. It should also be noted that repeated injection at suitable intervals into the same animal of a dead vaccine of the apyrogenetic type does not produce fever. This observation seriously affects the question of anaphylactic fever in the sense employed by Continental observers. We have gone more fully into this subject in a paper read at Liverpool in the summer of 1912.[2]

In the face of the experiments referred to here,[3] it must be admitted that if some laboratory-grown organisms appear to contain a protoplasmic pyrogen—because they produce immediate fever when injected dead—it is difficult to understand why so many others do not.

In further experiments, however, published[4] in the autumn of 1912, we showed that certain strains of organisms which after growth on agar are primarily apyrogenetic when injected dead appear to acquire pyrogenetic properties if subcultivation on fresh agar is carried out. For example, some subcultured strains of *S. aureus* readily exhibit the power of provoking fever if the heated organisms be injected. This is particularly the case if animal passage has taken place before subcultivation, the rabbit being selected as the medium of passage. In other words, subcultivation on laboratory media, especially if preceded by passage, appears to select out a race of organisms trained to extract from the culture medium a pyrogen that the original strain either could not extract or had become incapable of extracting. Control observations showed that the uninoculated media used produced no fever in the case both of agar and of broth. The suggestion therefore arises that when the subcultivated heated organism is injected pyrogen extracted or synthetized from the medium by the action of the living organism is unavoidably injected at the same time. It is possible, therefore, that the pyrogenetic properties of all those organisms injection of which when dead produces fever are in reality due not to the possession of pyrogenetic properties of their own, but to adsorption of something elaborated by the action of the living organism on the medium of growth. As will be seen, it has so far not been possible to detach completely this substance from organisms that have picked it up, possibly owing to its inclusion in interstices in the bacterial wall. Adsorption on the free surface of the bacterial wall appears not to prevent partial detachment of this adventitious pyrogen, since its presence can readily be demonstrated in the washings by the fever that follows their injection. If this theory—that the pyrogenetic effect of dead vaccines is due merely to adsorption of some substance manufactured from the medium of growth—is true, we ought to find that an inoculated medium when it has been deprived of its organisms is still pyrogenetic. And experiment proves this to be the case, provided that the centrifuge is used as the means of separation and not the bacterial filter.

A large number of observations have from time to time been made on the toxicity of bacterial filtrates expressed in fever.[5] And numerous observers have shown that many filtrates are practically non-toxic and produce no fever. At the same time it was shown that the heated material held up by the filter was often in these cases found to be strongly pyrogenetic as well as the heated unfiltered cultures. These findings naturally gave additional support to the view that the fever of infectious disease was caused by dead bacterial protoplasm. As, however, we showed[6] in 1911 the use of the filter is quite inadmissible in determining the presence or absence of pyrogenetic substances in culture media. And for two reasons. In the first place, it is difficult—if not impossible—to avoid contamination of the filtrates with antipyretic salts derived from the filter itself. Calcium salts, for example, were, we showed, traceable in the filtrate even after repeated passage of freshly distilled water. And calcium salts have been shown by many observers to inhibit fever. In the second place, although in view of our observation that pyrogen appears to be a body of small molecule[7] there is no guarantee that filtration of a colloidal liquid containing bodies of small molecule as well as of larger molecule, such as bacteria, will allow the filter to hold back the large and let through the small. On the contrary, larger bodies must tend to block the filter and prevent to an undetermined extent passage of the smaller.

Furthermore, apart from the question of filtration, if we wish to demonstrate the presence or absence of pyrogen by injection, it is always necessary to be sure that a sufficient volume of pure water is used as the diluent or vehicle of injection. This is necessary because increase of the volume of the diluent increases the amount of fever produced by injection of pyrogen, whilst injection of the same quantity of pyrogen in a smaller volume of diluent may produce no appreciable effect. Again, if normal saline be used as diluent instead of water, injection of one animal may produce high fever, whilst injection of a second animal of the same weight with the same quantity of pyrogen in the same quantity of saline may produce none. Experiments[8] dealing fully with all these points we have published elsewhere, though at present it is not possible to explain them. All observations, therefore, that deal with the pyrogenetic properties of bacteria, or of emulsions of bacteria, must be regarded as suspect (*a*) if the filter is used, (*b*) if saline be employed as the diluent, (*c*) if the relation of the volume of pure water and of the quantity of pyrogen containing material to body weight be not clearly stated, (*d*) if the water used for injection be not freshly distilled each day and used at once. The numerous fallacies introduced into the study of fever by absence of knowledge as to the necessity for this last precaution we have fully dealt with in other papers.[2 3 4 7] Here it will be sufficient to point out that the experimental evidence hitherto relied on in favour of the existence of such distinct entities of fever as water fever, salt fever, salvarsan fever, lactose fever, glucose fever, saccharose fever, protein fever, nuclein fever, tissue fever, fibrin ferment fever, serum fever, blood cell fever, haemolytic fever, transfusion fever, and anaphylactic fever no longer exists, since we have reproduced fever charts typical of all of these by injecting pyrogen-containing water alone, whilst the injection of each of these substances in pure water produced no fever.

Hence the only satisfactory method of determining by injection if an infected culture medium, after separation of the infecting organisms, is or is not toxic in the sense of producing fever is to use an efficient centrifuge and to employ as diluent freshly-distilled water in constant and stated volume. When these precautions are taken, the reputed atoxicity of many organism-free culture media that have been submitted to infection entirely disappears. The results of the large number of experiments we have carried out on this subject may be briefly illustrated.

Method of Experiment.

An eighteen hours culture, for example, of a stock strain of *B. typhosus* on broth is examined in the following ways:

A. *Unheated.*

1. A measured quantity—sufficiently small not to kill—of the whole broth is injected intravenously in a suitable quantity of water into a healthy rabbit of known weight. Fever rapidly

follows, reaching its maximum in two to four hours, and disappearing in six to eight hours. Observations are taken every thirty minutes. Observations taken at much longer intervals than this are useless. The next day and on several successive days observations are again repeatedly taken, but fever does not return within the limits of observation.

2. The centrifuged deposit of an equal quantity of the same culture is repeatedly washed and is then injected in water into a second animal, and observations are taken as before. The results are precisely the same as in the first experiment, allowing for the slight variations in the degree of fever produced as a result of the varying personal equation on the part of the animals injected.

3. The supernatant fluid not injected in Experiment 2 before washing is now injected in water into a third animal. Once more the same essential features are recorded.

B.—*Heated (to 100.0 C.).*

Precisely the same experiments described under A are now repeated with the heated culture, the heated centrifuged and washed organisms, and with the heated supernatant broth. And again the same results are recorded in every case. Examination of the heated supernatant broth shows that no organisms are present.

The heated and unheated centrifuged deposits from a fresh broth culture of the same strain are then washed with a solution of hydrogen peroxide. This procedure entirely removes their pyrogenetic properties, as was shown by injection into several animals. Microscopic examination of stained films of the deposit before and after this treatment reveals no differences. The heated and unheated centrifuged deposits from fresh samples of the culture are repeatedly washed with pure water, and the washings pooled. The washings produce on injection slight fever, the deposits produce high fever which is immediate. The heated and unheated centrifuged deposits from yet other samples of the culture are repeatedly washed with 1.4 per cent. sodium citrate solution in pure water and the washings pooled. These produce more fever than do the water washings. The deposits, however, are still markedly pyrogenetic. Finally, the heated and unheated centrifuged deposits from the remainder of the culture are repeatedly washed with pure ether and the washings again pooled. These are then evaporated in a water bath and the residue taken up in citrate solution and injected. The procedure is followed by high fever. The original deposit of organisms, however, is still somewhat pyrogenetic, possibly because it is not easy to get rid of all the other soluble pyrogen present. Owing to contamination of the bottom and sides of the test tube with the residue of pyrogen left after evaporation of the last remnants of the ether unavoidably left behind in removal of the supernatant fluid this appears to be the probable explanation. Examined by the microscope the ether washed organisms appear intact but moniliform, and they stain badly.

The points brought out by these experiments with ordinary laboratory strains may be thus epitomized:

B. typhosus grown in broth produces, if the heated or unheated organism be injected, an immediate and fugitive fever characterized by a sharp rise and fall. The animal does not become infected after injection of the living organism, and does not show any late fever after injection of either the living or the dead organism. Precisely the same events take place on injection of the culture medium after separation of the organisms. The pyrogen therefore is present in the separated broth and is at on the surface of the bacteria. This pyrogen cannot be completely washed away from the organisms, but it can be better detached by citrate solutions than by pure water, and better still by ether. It is, in short, feebly soluble in water, more soluble in citrate solution, and markedly soluble in ether. It cannot therefore, be a protein. It is apparently oxidized by peroxide since washing with this subject renders it inert, and standing for twelve hours in the semi-dry state has the same effect. We find in addition that it is active after incubation with a strong solution of tryptic ferment. Chloroform appears to some extent to inhibit its activity, but it is difficult to determine if this action is reversible owing to the difficulty of accurate determination of relative degrees of fever in the rabbit.

This pyrogen appears to be a preformed pyrogen when injected and to bear in its effects no relation to any known form of infection-fever occurring naturally in man and animals. After the immediate fever has passed off there appear to be no after-effects and there is no secondary fever, and this is also true after injection of the living or dead organism that has been allowed to stand in minimal quantities of water or saline. The pyrogen is, in addition, remarkably heat stable.

Such are some of the characteristics of a pyrogen that has hitherto been believed to be the purely bacterial product of a laboratory-grown organism that is known not to be pathogenic to the rabbit. Clearly, then, *B. typhosus* is not an organism in which it is possible to study natural pyrogen as it occurs in the body. Examination of other strains of *B. typhosus* on broth produced the same results, as also did examination of this organism on agar.

In order, therefore, to determine whether the pyrogen of infective disease is or is not a true bacterial product it is necessary to select for examination an organism that is pathogenic to the rabbit, and which at the same time has not become contaminated by a preformed pyrogen derived from the medium employed in artificial culture.

Certain strains of *S. aureus* satisfactorily meet these conditions. The absence of contamination in such strains is readily proved by injection. If, for example, the heated or unheated agar or broth culture be injected without separation of the organisms from the agar scrapings or broth no immediate fever is produced. The same negative result is obtained if the washed centrifuged organisms, or the supernatant fluid in the case of broth, or the washings in the case of agar, are injected. If, moreover, repeated observations are taken for several days of all the animals injected, it is found that fever only occurs in those which have received the living organism. In these animals, provided that the dose is not large enough to produce early death, or small enough to prevent infection, fever appears in a day or two, and continues till death or recovery takes place. If the virulence of the organism injected has not been reinforced by recent subcultivation, or by subcultivation *plus* animal passage, recovery is the rule. The fever generally begins within twenty-four hours of injection, but the latent period — if avirulent strains have been selected—was well marked in all the numerous cases examined. Here, then, is a very different picture to that produced by injection of *B. typhosus.* The fever set up is late in appearing instead of early, occurs always after injection of a suitable number of the living organisms, and never after injection of the dead; it has a slow instead of a rapid rise, and continues for several days instead of from one to eight hours. Further, it is not provoked by injection of the culture media from which the organisms have been separated, in marked contrast to the result obtained by injection of *B. typhosus* cultures deprived of that organism. The fever curve produced is that of a natural infection instead of one that bears no resemblance thereto. And finally, the substance—enzymic or what not in nature—which in the living organism liberates or synthetizes pyrogen is heat labile, whereas the pyrogen derived from laboratory media is remarkably heat stable.

Nevertheless the experiments so far quoted do not reveal the true source of the pyrogen in actual infections. A laboratory product, of course, natural pyrogen cannot be, as appears to be the case in the artificially produced fever set up by injection of cultures of *B. typhosus* and other members of the same group. Since, therefore, the source of natural pyrogen is not dissolved bacterial protoplasm, it can only be the substrate offered to the living organism by the tissues of the host.

The next step in the solution of the problem, therefore, was to discard laboratory media and to employ, as far as possible, the natural media of the animal body. For this purpose red cells from the blood of healthy rabbits were washed free of plasma with pure solution of sodium citrate, and were at once lysed in freshly distilled water. Samples of this blood-cell extract medium were exposed for one hour to air infection, and were then placed in a sterile flask, which was at once hermetically sealed and then incubated for twelve hours at 37.0° C. At the end of this time a copious growth of a staphylococcus was present, and demonstrated by film preparations from the centrifuged deposit. Control blood cell extract medium prepared at the same time and treated in the same way, with the exception of exposure to air, was found to be sterile. Injections of this control extract were followed by marked depression of temperature. Injections were then made of the infected extract into different animals as follows: (*a*) The heated washed centrifuged organisms; (*b*) the unheated washed organisms; (*c*) the unheated supernatant blood cell extract proved by control inoculations of agar to be free from organisms capable of growth. In each case immediate fever resulted of a precisely similar nature to that obtained by injection of *B. typhosus* cultures or of staphylococcal cultures after subcultivation preceded by passage. The animals injected with the heated organisms or with the supernatant liquid on the other hand failed to show any secondary fever. The animals, however, that were injected with the living organism exhibited in addition to immediate fever the late continued fever of a natural infection with a much shortened latent period.

From these experiments it is clear that a pyrogen clinically indistinguishable from the pyrogen elaborated from agar or broth can be readily obtained if blood-cell extract be used as the medium. It is also clear that the immediate fever produced after injection of the heated organism or the supernatant fluid or the unheated organism was due to injection of preformed pyrogen—elaborated from the blood extract in the test tube—whilst the late fever produced after injection of the living organism was due to a second pyrogen—elaborated from the circulating blood or other tissues. The fact that the organisms were obtained direct from the air excludes the possibility of transference of any contamination from the ordinary laboratory media. These experiments appear to support the view that an organism that is unable to produce immediate fever dead or alive can do so both dead and alive if the living organism is first able to extract and adsorb from the medium on which it grows a pyrogen elaborated from that medium. We have elsewhere shown that organisms derived from the air, and not allowed to grow on the ordinary laboratory media, produce no immediate fever on injection provided that pyrogen-free water or saline is employed as the vehicle of injection. The pyrogen of blood-cell extract is just as heat stable as is the pyrogen derived from agar or from broth.

Owing to individual variation in the degree of fever produced in rabbits even after injection of constant quantities of pyrogen in constant quantities of water relatively to body weight, it has not been found possible accurately to measure the increase of "test tube pyrogen" from day to day in living cultures. The concentration, however, of "test tube" pyrogen is certainly high after twelve hours' growth, and remains high, in broth cultures, for many weeks. Abstraction of pyrogen taking place spontaneously is, of course, impossible, and no great destruction occurs within reasonable limits of observation. On the other hand, in the living body, during a natural infection we should not expect to find the same conditions. The concentration of pyrogen in the circulating blood of a feverish subject, for example, should be relatively low, since abstraction, if not destruction as well, must continually be going on. We should expect, therefore, to find that in order to produce fever in a healthy animal by injecting blood from a feverish animal it would be necessary to inject large quantities of blood. And on experiment this proves to be the case. For example, I injected into a series of healthy animals varying quantities of a 40 per cent. centrifuged citrated blood plasma from an animal exhibiting the continued fever of an acute genuine infection. The mixture of plasma and 1.4 per cent. citrate solution was centrifuged in order to get rid of any organisms that might be present. Injections up to 5.0 c.cm., equivalent to one four-hundredth of the weight of the animal injected, produced no fever. Injections of 10.0 c.cm. provoked slight rise of temperature, whilst injection of 15.0 c.cm. produced slight but definite fever. Control injections of healthy citrated plasma produced no rise of temperature. Clearly, therefore, the amount of pyrogen circulating in the blood in such case is much less than that contained in blood-cell extract in the test tube, which only requires injection of 0.50 c.cm. of a 25 per cent. extract, from which the organisms have been separated by the centrifuge, to produce high fever. The next step, therefore, was to determine if the injection of heated organisms after recovery from the body of a feverish animal does or does not produce immediate fever. If it were found that injection of such organisms did produce an immediate fever similar to that produced by injection of heated organisms after test tube growth on agar or broth or on blood cell extract, the fact would appear to make demonstration of the true source of pyrogen in infective disease by this method of investigation an almost impossible achievement. If, on the other hand, injection of heated organisms recovered from infected subjects produced no fever whatever the number injected, the theory that dissolved bacterial protoplasm is the primary source of the pyrogen of infective disease would be clearly untenable. Demonstration of this would, in conjunction with the experiments already described, leave no alternative but acceptance of the view that fever is the expression of the activity of living organisms, or their products, in and on the infected body.

Further experiments were therefore carried out (*a*) with organisms recovered from the fixed tissues of infected subjects, including pus, (*b*) with organisms recovered from the blood in subjects of septicaemia.

Recovery of Organisms from Infected Tissues.

Organisms were recovered from infected tissues in the following way:

An unheated laboratory strain of S. *aureus* that produced no immediate fever was injected into a series of healthy rabbits. A late continued fever resulted in all cases. After a week's illness with fever one of the animals was killed, and from the multiple abscesses present in the kidneys large numbers of organisms were heated before injection to 57° C. No fever whatever followed the injection early or late. The experiment was repeated with another animal which was killed at an earlier stage of the infection and the same results were obtained. In another experiment an unheated laboratory strain of a pneumococcus of low virulence was injected into a healthy rabbit. No immediate fever followed injection, but a late fever set in. On the eighth day, the fever still continuing, the animal was killed. An emulsion of splenic pulp, shown by film preparations to contain enormous numbers of organisms, was injected. Again no fever followed injection. In this experiment, as well as in the first series, control cultivations from the kidney and spleen showed that living organisms were present in the material examined, since copious growth was obtained on agar.

Injection of the heated and unheated agar organisms produced high immediate fever in every case, and the injections in the case of the living organisms led next day to a fatal issue.

These observations therefore appear to show that organisms recovered from tissues other than the blood, and injected at once, possess no pyrogen, but that when grown on laboratory media they readily elaborate a contaminating pyrogen from such medium, which they subsequently adsorb. They also show that the concentration of body pyrogen cannot be high in the infected organs, since the tissue material injected in some of the cases was considerable in amount. Owing to the difficulty, however, of freeing organisms recovered in this way from tissue débris, their enumeration cannot be satisfactorily carried out. Moreover, it is conceivable that absence of pyrogen in the dead organisms after recovery from the body might be explained by exhaustion of any pyrogen they might have contained whilst in the body during the continuance of fever. The low concentration of pyrogen, however, already demonstrated in the blood plasma during high fever, and the fact that injection of organisms recovered from the body at an earlier stage of an infection shows them also to be pyrogen free, make such a view improbable.

Fresh Human Pus.

Pus was first taken from an abscess believed to be tuberculous in a patient with a temperature of 100° F. In film preparations no organisms could be found. Intravenous injection of small quantities of this pus in water was not followed by fever, early or late, whether heated or unheated. Samples of fresh pus were then taken from several patients with fever, and were examined bacteriologically and by intravenous and subcutaneous injection. Only those specimens were selected for injection which were found to contain large numbers of micro-organisms indicating the presence of recently-formed pus, or of pus from the periphery of abscess sacs. Control cultivations on agar gave in all cases selected copious growth. The organisms injected in different specimens included staphylococci, diplococci, and streptococci. One specimen contained both staphylococci and streptococci. In no case did injection of quantities of heated pus from 0.1 gram in 1 c.cm. of water to 2.50 grams in 2.50 c.cm. of water produce fever early or late. On the other hand, injection of sufficient quantities of unheated pus in this series never failed to produce a continued fever with a much shortened latent period.

These experiments are of interest in view of the numerous observations that have been published to show that injection of pus is constantly followed by fever. Such observations can only be explained by injection of pus in pyrogenetic saline, or by injection only of unheated pus. The experiments here quoted, however, lend additional support to the view that fever can only be produced during a natural infection by the living organism. In the case, however, of pus injections it is again impossible to carry out satisfactory enumeration of the organisms injected, and this method was therefore abandoned.

The only convenient method remaining of determining whether organisms recovered from the body during an infection with fever do or do not produce immediate fever when injected dead is to obtain them direct from the blood stream in cases of septicaemia. If the circulating blood in septicaemia be received into pure sodium citrate solution and the mixture at once centrifuged, the deposit can, after lysis of the red cells in pure water, be readily examined. Any organisms present can then be counted in the ordinary way. In laboratory experiments, however, as is well known, it is a difficult matter to produce a genuine bacteriaemia, since the injection of organisms after growth on ordinary laboratory media is followed by their rapid disappearance from the blood stream as estimated by ordinary cultivation methods. And the cultivation of serum-fast strains has certain disadvantages. I have, however, overcome these difficulties by growing, for example, a laboratory strain of pneumococcus on the rabbit blood cell extract above described for forty-eight hours. Injection of the living organism from such extract into a healthy rabbit produces an infection with fever that can be demonstrated for some days. And injection of known numbers of organisms recovered from the blood during this fever can be readily carried out. Injection of 3,090 million dead organisms recovered in this way failed to produce any fever early or late, whilst injection of 10.0 c.cm. of a 40 per cent. citrated solution of plasma from the same animal, after removal of any organisms present by the aid of the centrifuge, produced slight but definite rise of temperature, and injection of the living organisms produced a typical continued fever.

Demonstration of the absence of pyrogen in dead bacterial protoplasm of organisms recovered during natural infections appears therefore in the cases examined to be complete. Whether this will prove also to be true of all organisms recovered from infected subjects, including man, is a question that only further study can decide.

In the meanwhile, in view of the cumulative evidence here presented, it is difficult to resist the conclusion that a rising temperature is not—as we have paradoxically been led to believe—a sign of increase of bactericidal and bacteriolytic function, but is on the contrary an expression of the activity of living organisms. Dead organisms, in short, do not in natural infections give rise to fever.

The next question that arises is the practical bearing of these observations on immunity problems in general and in particular on the administration of dead bacterial vaccines in the diagnosis, prevention and treatment of infective disease.

It will, I think, be clear that the non bacterial pyrogen derived from laboratory media which is so misleading a factor in the experimental study of fever is also one that we cannot afford to ignore in practical medicine.

There are in connexion with prophylactic and therapeutic vaccines two possibilities to be considered. On the one hand contamination of bacterial vaccines with pyrogenetic substances of purely laboratory origin may greatly enhance their prophylactic and therapeutic value. In this connexion the observations made some years ago on the effect of injecting nucleinates are of interest, though they cannot be dealt with here. If comparative experiments with the vaccines at present in use and with detoxicated vaccines prove that the presence of non-bacterial substances is an advantage, steps will have to be taken by subcultivation or in other ways to ensure that all vaccines are toxic. At present some vaccines are toxic in this respect, and some are not. Some of the causes for these differences I have explained. On the other hand, contamination of vaccines with laboratory material may be a distinct disadvantage. If this prove to be the case, vaccines, before they can be justifiably used in man, must first be tested for the absence of medium derivatives by injection of animals with the precautions mentioned, or in some other way. And such vaccines as are found to be toxic must then be rendered atoxic by one or other of the methods given, or by some other method if shown to be more convenient. It will not be forgotten that the injection of contaminated vaccines into healthy subjects for purposes of prophylaxis may be a less serious affair—if disadvantage be proved—than therapeutic injection of subjects already gravely ill. In these the question of dosage will necessarily be a matter of special concern, unless vaccines shown to have been detoxicated, or to be primarily atoxic, are employed.

Of more importance, however, even than the question of the positive good or harm effected by injections of contaminated vaccines is the larger question of to what extent the specific antigenic values of vaccines in general depend on the presence of this contamination. (In passing it may be well to point out that there is some evidence that the toxic substances elaborated from culture media by the action of living organisms on such media are to a certain extent specific to those organisms.[*])

In other words this problem presents itself: Have we at present unimpeachable evidence that a pure dead vaccine has any antigenic values in the prophylaxis or treatment of general or local infections? It is worth notice that the vaccines for which the highest protective and therapeutic values are claimed are just those in which the content of laboratory pyrogen is highest. Cases in point are *B. typhosus*, *B. coli*, possibly *B. pestis*, and the acne bacillus. I am not here suggesting that this relation is more than accidental, or that dead vaccines have no specific antigenic values apart from their laboratory contamination. It is obvious, however, that until the point has been fully investigated we are no longer justified in attributing to dead bacterial vaccines antigenic values that may prove to belong—in whole or in part—to substances that are not primarily derived from dead bacterial protoplasm. At present when we employ these vaccines we have no guarantee that any single vaccine provided is a pure vaccine.

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[*] For example, the centrifuged washed organisms of *B. coli* laboratory cultures cannot be deprived of their pyrogenetic properties by washing with hydrogen peroxide. This fact appears therefore to indicate that the living *B. coli* elaborates from its culture medium a pyrogen of a different nature to that elaborated by the living *B. typhosus*, Moreover, *B. coli* is able to synthetize a pyrogen from protein-free media which before inoculation with the living organism are apyrogenetic.

In future, therefore, we shall require such guarantee, as well as an assurance that the process of purification has not damaged any genuine antigen the purified vaccine may possess. And in passing it may be well to glance at the question of the so-called autogenous vaccine, which in one important respect is a somewhat misleading name, as I pointed out in 1911. A vaccine made from dead organisms collected from the blood or other tissues of an infected subject and injected without intermediate cultivation on laboratory media is a genuine autogenous vaccine, and its injection in animals appears not to be followed by toxic symptoms, though it remains to be seen if it has any therapeutic effect. A vaccine, however, that is made from organisms collected in the same way, but then cultivated on artificial media and then injected—after death of the organisms has taken place—is not a genuine autogenous vaccine, because, as I have shown, the toxic symptoms that may now arise are due to contamination from the laboratory medium of growth. The organism, in short, has had a new function imposed upon its descendants by such growth, and it is not impossible, therefore, that the increased therapeutic values with which the *soi-disant* autogenous vaccine is credited may prove to be due to this very fact.

It is not stretching the argument too far to point out that it is also necessary in view of the experiments here described to examine afresh the evidence that has hitherto been relied on in favour of the existence of bacterial toxins in general and especially of the so-called endotoxins. The endotoxin of *B. typhosus* is a case in point. In all these cases it will now be necessary before we can postulate the existence of a genuine endotoxin to be sure that the organism selected for purposes of extraction, whatever the method of extraction selected, is first of all deprived of the highly toxic material elaborated from the culture medium. In all experiments so far undertaken with the reputed toxin of *B. typhosus* when cultivated on laboratory media this precaution has for obvious reasons not yet been taken.

Finally comes the question of diagnosis. If, for prophylactic or therapeutic purposes, it is necessary to use pure vaccines, it is equally so in all those test tube reactions in which antigen and antibody are the essential factors. We shall not only have to make certain that only pure vaccines are used for the production of animal immune serums, but we shall also have to ensure that all bacterial emulsions used as diagnostic antigens are equally above reproach. In the case of such organisms as the bacillus of Koch, and of such other members as apparently do not elaborate this contamination from culture media these precautions may prove unnecessary. On the other hand, in the case of all those organisms which we have shown to be characterized by their ability in the living state to elaborate toxic derivatives from the media of artificial growth, these precautions cannot be neglected.

In conclusion, although it is premature to indicate the practical lines of direct immunity research opened up by these experiments, it is impossible to ignore the suggestions they offer as to the meaning of sensitization and the superiority of living vaccines over dead, as well as to the necessity for re-examination of the whole scheme of vaccine-therapy. And in the meanwhile, if they do no more than press home the necessity for fresh investigations into the true source and character of the natural antigens of disease they will faithfully have served their purpose.

Note.—Since this paper was placed in type two articles on experimental fever have appeared in a German periodical by Thiele and Embleton which might seem at first sight to affect some of the conclusions reached in this communication.[9] In the articles referred to, the authors—as so many have done before them—have fallen victims to the very fallacies which Dr. Penfold and I have already exposed in the English journals.[10] One of the fundamental errors, for example, in all study of experimental fever produced by injection is the assumption that water which is innocent of demonstrable infection at the time of injection must also be innocent of pyrogen, and that water collected direct from the condenser of a distilling apparatus in daily use and then filtered is necessarily pure water. In consequence, therefore, of failure to recognize this source of error these workers in many of their experiments unwittingly used impure water, as is clearly shown in their own protocols. This accident is alone sufficient to invalidate all the experiments and deductions based thereon, though there are other fallacies of almost equal importance which I have fully described elsewhere.

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[3] Hort and Penfold: Micro-organisms and their Relation to Fever, *Journ. of Hygiene*, vol. xii, No. 3, October 21st, 1912.

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[8] Hort: Normal and Immune Serum-Therapy, British Medical Journal, November, 1911.

[9] *Zeitschrift für Immunitätsforschung*, 1913.

[10] The Relation of Salvarsan Fever to other Forms of Injection Fever. *Proc. Roy. Soc. Med.*, 1912, vol. v (Path. Sect.), p. 137. Chart.

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MICROORGANISMS AND THEIR RELATION TO FEVER[1].

(Preliminary communication.)

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(*From the Lister Institute, London.*)

(With 27 Charts.)

It appears to be widely believed that the fever of infective disease is due to absorption of pyrogenetic substances liberated from dead microorganisms by a combined process of lysis and extraction in the body of the infected subject. The diffusion of products of the action of bacterial autolytic enzymes on microorganisms after their destruction by the defensive agencies of the body is a variant on the same theme. Closely allied are the theories of protein fever, and of anaphylactic fever, in so far as they relate to the supposed possession by microorganisms, or their split products, of pyrogenetic properties. The absorption of alien protein, or of derivatives of alien protoplasm, from organisms that are dead, is, in short, the essential feature of this conception of fever production.

At present there is no satisfactory evidence available to support this theory. On the contrary, there are many clinical facts in the way of its acceptance, though their interpretation is a matter of great difficulty.

In 1909 one of us therefore suggested(1) as an alternative view that the source of fever in infection may be, not the dead cells of the invader, but the tissues or fluids of the host. In other words it is possible that fever may be an index, in infective disease, not of the death and lysis of causal organisms, but of the ability of the living organism, or, if it be

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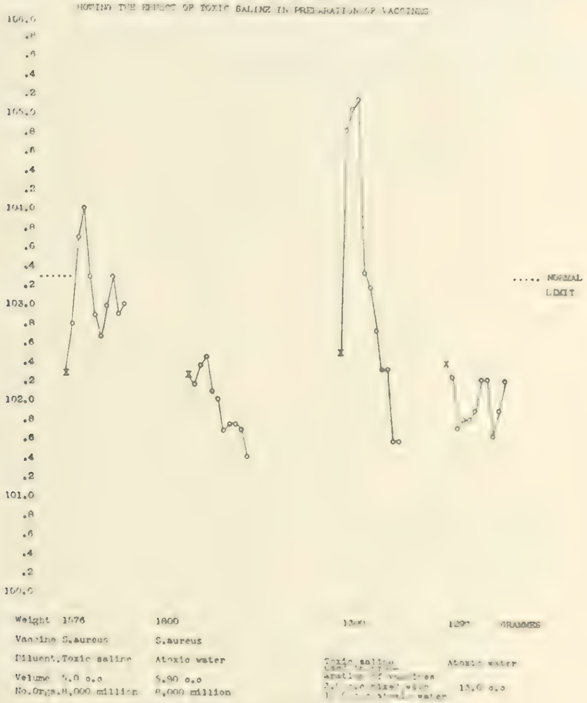
[1] The experimental expenses of this research were defrayed by the Constance Research Fund administered by one of us (E. C. H.). The authors wish to record their thanks to the Council and Directors of the Lister Institute for the laboratory facilities placed at their disposal.

damaged, of its derivatives, perhaps enzymic in nature, to liberate from the natural medium on which it grows pyrogenetic bodies that it does not itself possess. This might be brought about in one of two ways. The pyrogen might be a product of the action of autolytic enzymes of the cells of the host on the substrate provided by these cells after their destruction by the toxic agents of microorganisms, or it might be a product of the action of bacterial enzymes on the same substrate. In either case the essential factor in the production of fever would be the liberation of pyrogenetic substances of native origin, as opposed to the alien source of supply suggested by the generally accepted view.

Thus broadly stated these two theories as to the source of pyrogen in infective disease are mutually antagonistic. In order to make clear the points we wish to establish it is necessary to refer to certain fallacies in all experimental enquiry into the fever produced by microorganisms.

(1) We have recently shown that the injection into animals of various substances suspended or dissolved in saline or water may give rise to fever which depends, not on the nature of the substance injected, but on contamination of the diluent employed. Water or saline may be absolutely free from living or dead microorganisms at the time of injection, and may yet be strongly pyrogenetic. The contaminating principle in such water or saline we have shown (2) to be a heat stable body of small molecule, incapable of removal by ordinary methods of filtration, and bearing no relation to the degree of demonstrable infection of these liquids. This pyrogenetic substance may or may not be a derivative of bacterial cells, but whatever its nature its unsuspected presence has given rise to belief in many different forms of fictitious injection fever, such, for example, as water fever, salt fever, sugar fever, ferment fever, tissue fever, salvarsan fever and so forth. In the study of bacterial fever the same fallacy that we demonstrated in the forms referred to has crept in, and we find that many organisms of different types owe their apparent pyrogenetic properties to the fact that the saline used for purposes of suspension or solution has become contaminated with the body described. The cognate study of protein fever is, we now find, materially affected by the same unsuspected fallacy, which therefore directly affects the question of bacterial protein fever. And this is particularly the case in anaphylactic fever where multiple injections of bacterial or other protein suspended in water or saline must of necessity be given. Our charts will show that if precaution be taken to use water that has been freshly distilled the apparent pyrogenetic properties of certain organisms at once disappear. This is illustrated by Chart 1.

In the cases injected we have also been unable to obtain any evidence of anaphylactic fever. Our experiments on this subject will be dealt with in a separate paper. To what extent, however, our results affect the value of the evidence offered by Vaughan(2a), Wheeler(2b), Friedberger(2c), Schittenhelm and Weichardt(2d), and others on the subject of bacterial fever, endotoxin fever, protein fever and anaphylactic fever may be seen by reference to a paper recently read by us[1]. If this be studied in the light of the knowledge we now possess it will be realized how manifold are the chances of error introduced into all experiments of this nature involving the use of water or of saline of unknown origin. It is necessary to remember that demonstration of the sterility of these liquids by attempts at cultivation affords no guarantee of the absence of the heat stable pyrogen described.



Interval between observations 30 minutes.

Chart 1.

(2) There is a second source of error not yet fully recognized. This was introduced by Friedberger(3) when he selected the guinea-pig for his

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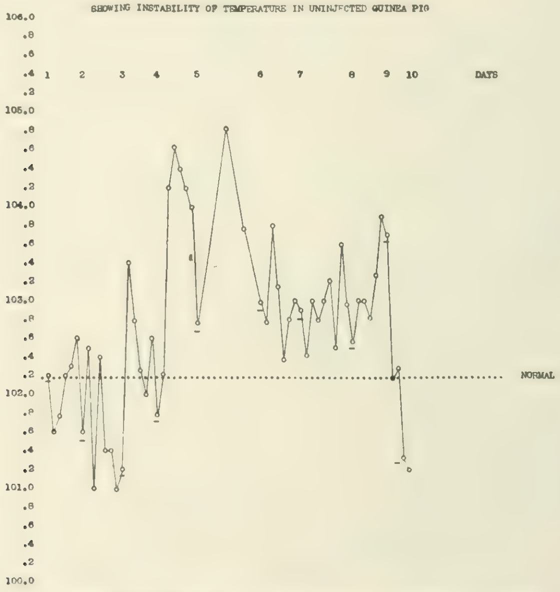
[1] Brit. Med. Assoc. Meeting at Liverpool, July, 1912.

experiments on anaphylactic fever. In this animal as Hort showed(4) in 1910 the temperature is so unstable, partly owing to the impossibility of avoiding injury to the rectal mucous membrane when taking thermometric observations, that it is impossible to draw safe conclusions as to temperature changes after injection. Chart 2 demonstrates the accuracy of this statement, as the temperature curve given is that of an animal which received no injection. In order to show that this was no chance observation records were taken for several days of six other animals. In all cases the same kind of results was obtained. In the rabbit fortunately this objection does not hold as our numerous controls will show.


SHOWING INSTABILITY OF TEMPERATURE IN UNINJECTED GUINEA PIG

INTERVAL BETWEEN OBSERVATIONS 60 MINUTES EXCEPT AT MARK –, WHERE THERE IS AN INTERVAL OF 12 HOURS

Chart 2.

(3) In spite of care taken to avoid these two sources of error we soon encountered a third of a totally unsuspected nature. We find that in determining the pyrogenetic properties of small fractions of a broth culture of *B. typhosus*, for example, increase of the volume of the diluent often has a striking effect in increasing the extent of fever produced. This is shown in Charts 3, 5 and 7. Moreover if a larger fraction of the culture, or even the same fraction, which when given alone is non-lethal, be injected in a volume of water equivalent to from

about 1/100th to 1/25th of the body weight death often ensues in an hour or two. This is shown in Charts 3, 6, 7 and 8. In Chart 9 are given control observations showing the absence of lethal effect in the case of undiluted cultures. Control observations as demonstrated in Chart 4, show that neither fever or death is ever produced by pure water alone after injection in these quantities. The former of these effects is the only one we can pursue here. In all these cases the cultures

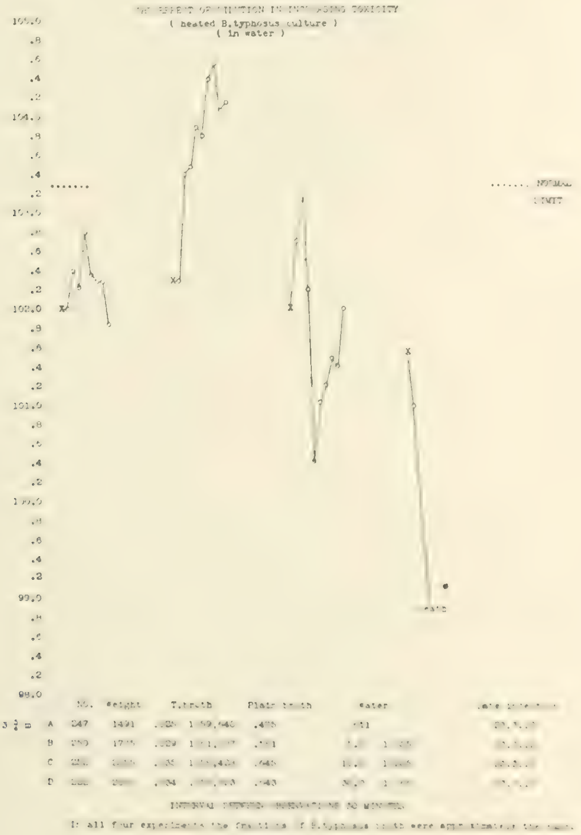
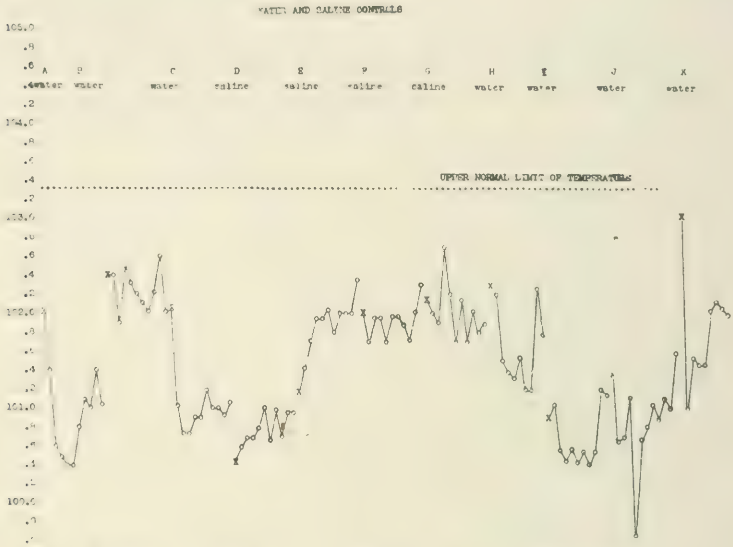


Chart 3.

were injected 18 hours after inoculation of the broth. The charts show the importance of recognizing this source of error as regards the interpretation of fever. At first sight it would appear that an explanation of this might be found in the facility afforded to *in vitro* extraction by the addition to a constant weight of bacterial culture of increasing quantities of distilled water. We find, however, that the same phenomenon may occur when normal saline is used instead of water. We also find that if water or saline be injected for some minutes before the fractions of culture are injected this greatly intensifies the effect, though the exact time limits of this curious form of sensitisation by water or saline have not yet been determined. This is shown in Charts 5, 6 and 7 as regards water. Clearly therefore the increase of toxicity induced by increase in the volume of diluent cannot be merely due to increased facility of extraction of toxic bodies in the test tube. We attempt no explanation, but are content for the moment to record the facts.



	No. of animal	Weight		Water in C.C.		Date of injection
A	360	1690		12.60	1/134	9.5.12
B	361	1620		12.60	1/134	9.5.12
C	362	1690		12.60	1/134	9.5.12
D	365	1633	SALINE	12.20	1/134	9.5.12
E	366	1350	SALINE	10.10	1/134	9.5.12
F	367	1350	SALINE	13.50	1/100	9.5.12
G	368	2250	SALINE	22.50	1/100	9.5.12
H	363	1746		17.46	1/100	9.5.12
I	364	1633		16.33	1/100	9.5.12
J		1690		18.0	1/ 93	17.4.12
K		2112		87.0	1/ 24	?

ANIMALS A to I all from one family (white)

INTERVAL BETWEEN OBSERVATIONS 30 MINUTES

Chart 4.

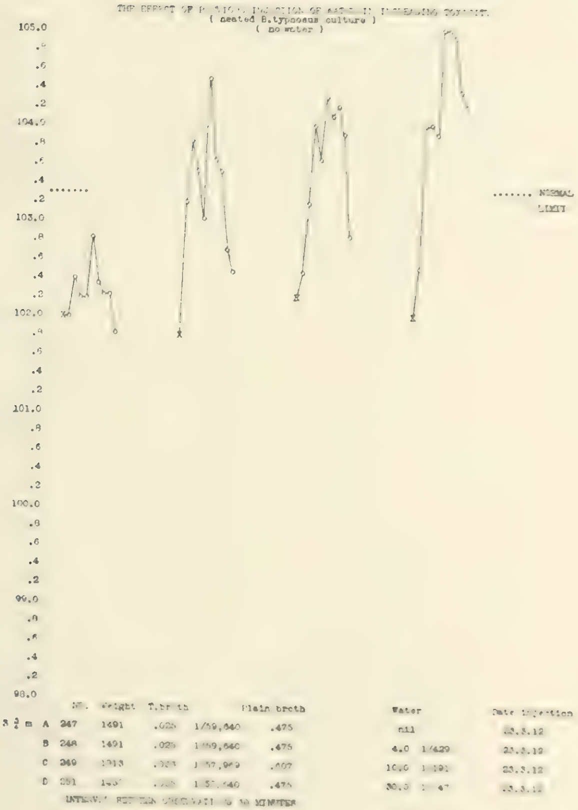
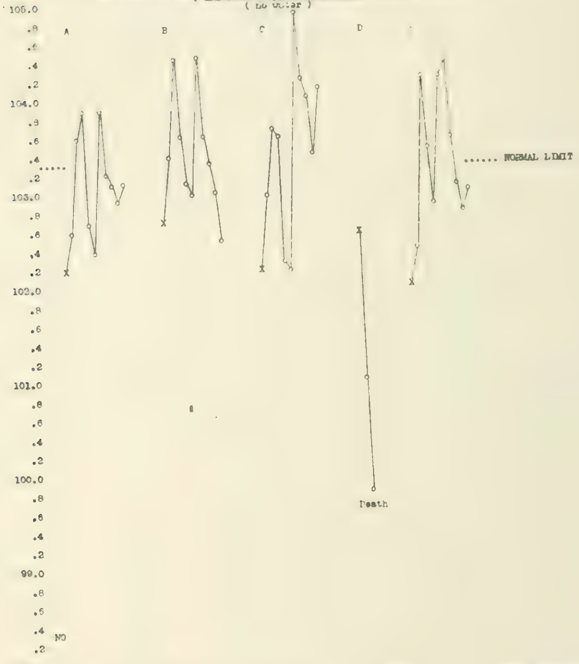

(heated B.typhosus culture)
(no water)
105.0
.8
.6
.4
.2
104.0
103.0
102.0
101.0
100.0
99.0
98.0
.... NORMAL LIMIT
Weight
Plain broth
Water
3 3/4 m
A 247 1491 1/59,640 .475 nil
B 248 1491 .025 .475 4.0 1/429 23.3.12
C 249 .607 23.3.12
D 251 .475
23.3.12

Chart 5.

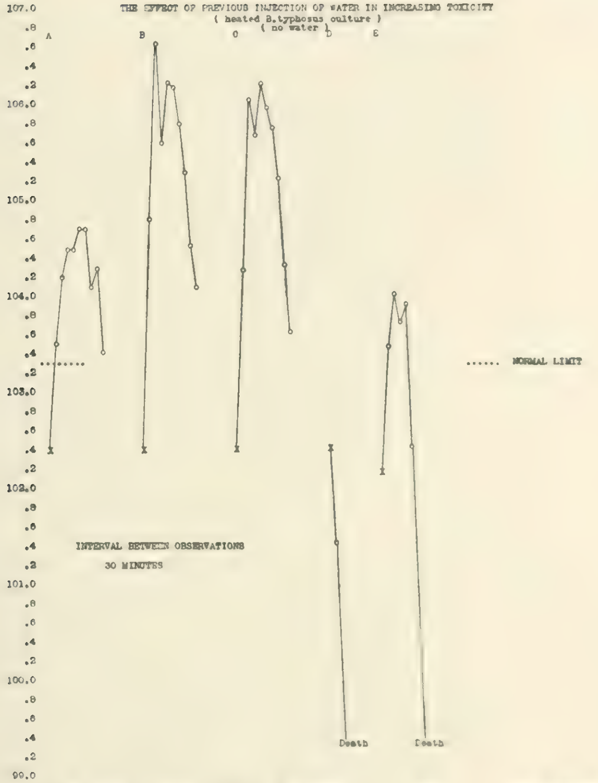
THE EFFECT OF PREVIOUS INJECTION OF WATER IN INCREASING TOXICITY
(unheated B.typhosus culture)
(no water)



		NO.	Weight	T.broth		Plain broth	Water		Date injection
5 m	A	274	2026	.032	1/63,312	.168	nil		17.4.12
5 m	B	275	2026	.032	1/63,312	.168	5.06	1/400	17.4.12
4 m	C	276	1633	.026	1/62,807	.137	8.10	1/200	17.4.12
3 m	D	672	1463	.023	1/63,607	.123	21.90	1/ 66	17.4.12
3 m	E	278	1463	.023	1/63,607	.123	36.50	1/ 41	17.4.12

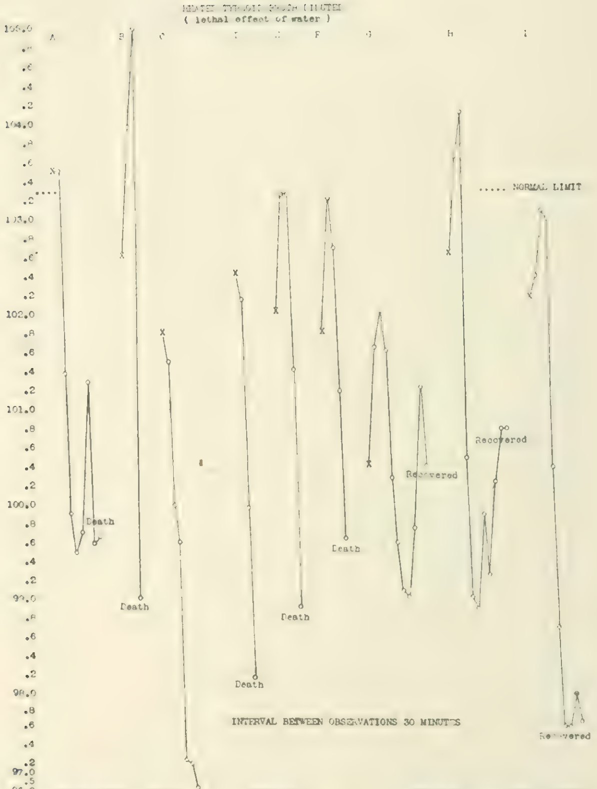
INTERVAL BETWEEN OBSERVATIONS 30 MINUTES

Chart 6.



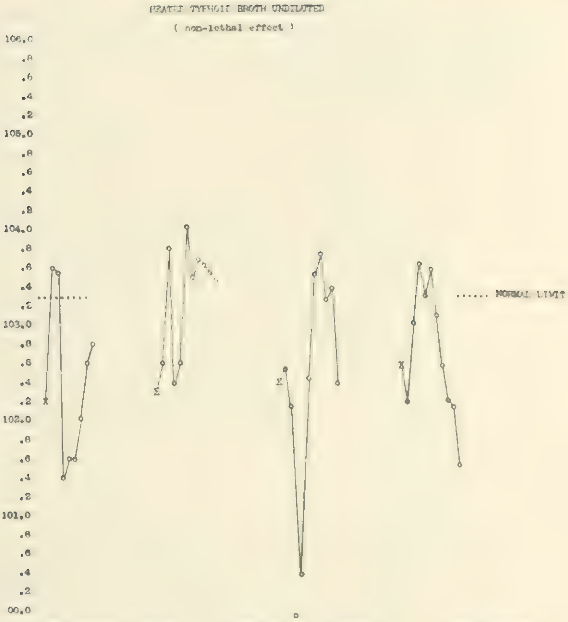
		NO.	Weight	T.broth		Plain broth	Water		Date injection
4 m	A	270	1690	.027	1/62,592	.133	nil		16.4.12
	B	271	1690	.027	1/62,592	.133	4.20	1/402	16.4.12
	C	272	1913	.032	1/59,781	.148	9.50	1/201	16.4.12
	D	732	1576	.024	1/65,666	.126	23.60	[illegible]	16.4.12
	E	731	1941	.030	1/64,700	.160	[illegible]	[illegible]	16.4.12

Chart 7.



	NO. of animal	Weight	Broth in c.c		Water in c.c		Date injection
A	353	2250	.67	1/3,373	16.10	1/139	6.5.12
B	153	2363	.88	[illegible]	[illegible]	1/100	[illegible]
C	317	[illegible]	.76	[illegible]	[illegible]	1/65	[illegible]
D	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	1/105	[illegible]
E	[illegible]	1520	.57	[illegible]	[illegible]	[illegible]	24.4.12
F							
G	[illegible]	2305	.88	[illegible]	23.00	1/100	6.5.12
H	[illegible]	1463	.54	[illegible]	10.60	1/137	24.4.12
I	[illegible]	1913	.47	[illegible]	[illegible]	[illegible]	23.4.12

Chart 8.



NO. of animal	Weight	Broth in c.c		Diluent	Date injection
312	1520	.58	1/2,620	nil	25.4.12
313	1549	.60	1/2,583	nil	25.4.12
316	1548	.60	1/2,580	nil	25.4.12
265	1633	1.60	1/1,088	nil	26.3.12

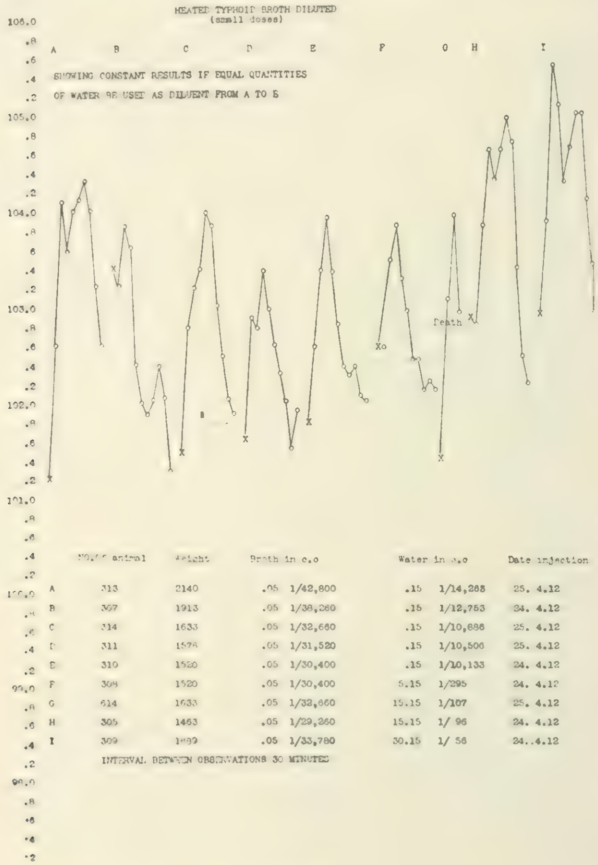
INTERVAL BETWEEN OBSERVATIONS 3. MINUTES

NOTE IN LAST EXPERIMENT THE MINIMAL DISTURBANCE OF TEMPERATURE CAUSED BY LARGEST DOSE.

THIS ANIMAL DIED IN 25 DAYS

Chart 9.

(4) There is another source of fallacy in connection with the diluent employed which is equally difficult to explain. If a suitable quantity of a heated or unheated culture of *B. typhosus* be injected, after suspension, in constant volumes, of distilled water, relatively to body weight, fever appears in all the animals injected. The extent of fever may vary in individual animals to a slight extent, but the reaction never fails.



	NO. of animal	Weight	Broth in c.c		Water in c.c		Date injection
A	313	2140	.05	1/42,800	.15	1/14,268	25. 4.12
B	307	1913	.05	1/38,260	.15	1/12,753	24. 4.12
C	314	1633	.05	1/32,660	.15	1/10,886	25. 4.12
D	311	1576	.05	1/31,520	.15	1/10,506	25. 4.12
E	310	1520	.05	1/30,400	.15	1/10,133	24. 4.12
F	308	1520	.05	1/30,400	5.15	1/295	24. 4.12
G	614	1633	.05	1/32,660	15.15	1/107	25. 4.12
H	305	1463	.05	1/29,260	15.15	1/ 96	24. 4.12
I	309	1689	.05	1/33,780	30.15	1/ 56	24..4.12

INTERVAL BETWEEN OBSERVATIONS 30 MINUTES

Chart 10.

We make this statement after injection of nearly two hundred animals with this organism. Examples are shown in Chart 10. On the other hand we find that if normal saline, ·85 °/₀, be injected in the place of the water most inconstant results follow in regard to fever. In a pair of animals for example injected with the same fraction of culture and the same volume of saline, relatively to body weight, one animal may show a rise of temperature to 105·0° F., whilst the other animal is scarcely affected. This is shown in Chart 11. This phenomenon we frequently noticed in our work on fictitious salt fever published last year. We noticed the same thing also 12 months ago when studying the relative toxicities of different kinds of toxic exudates dissolved in water or in saline. Recognition therefore of this source of fallacy is certainly necessary in studying experimental fever, and other indications of toxic action. In all the experiments here quoted distilled water that we have prepared each day for the purpose has been used as the diluent, unless otherwise stated.

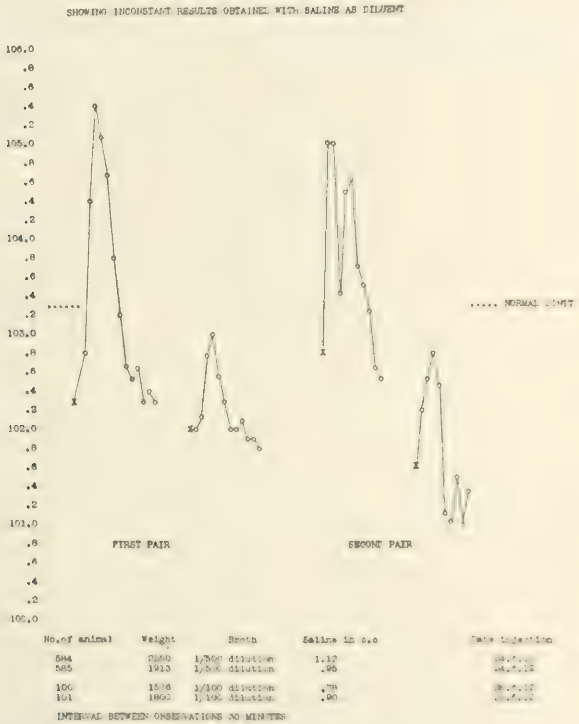
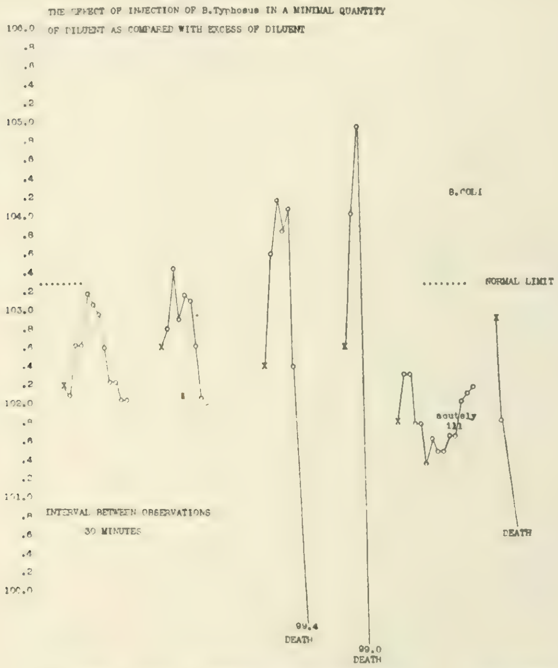


Chart 11.

(5) The last source of error we wish to refer to in connection with bacterial fever investigation is due to the fact that the effect may be masked if the dose given be too large. If a small but sufficient fraction of a *B. typhosus* culture be injected a fairly definite extent of fever is always produced if water be the diluent, as exemplified in Chart 10. If, however, a larger quantity of both culture and water be given a

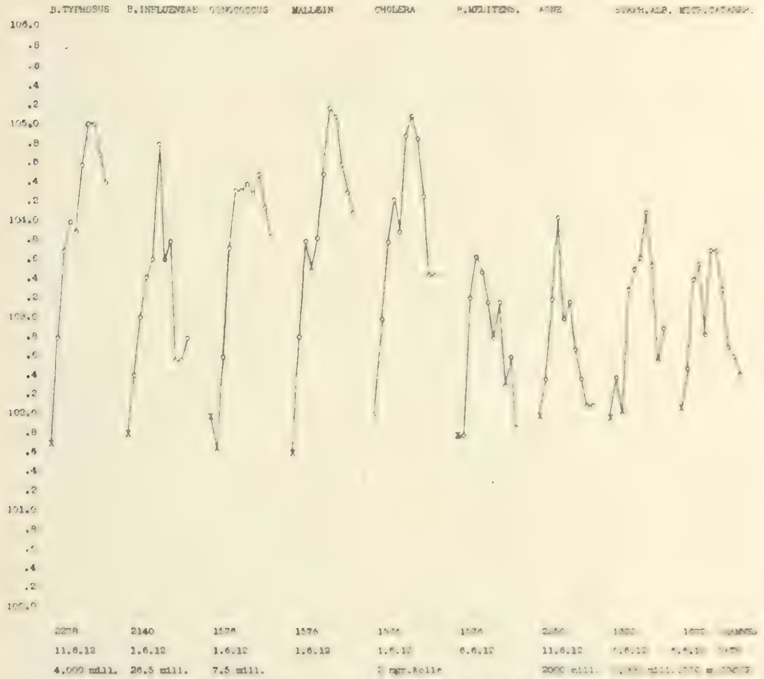
greater amount of fever ensues, as already described, up to a certain point. If this point be passed, as was seen in Chart 8, a large injection produces first a rise of temperature and then a fall, often of six or more degrees before death takes place. Recovery, however, may occur. The fall noted is either due to an excessive dose of the pyrogen producing an inverted effect, as has been often suggested though never proved, or it is due to the action of an excess of endotoxin or other depressant body. Owing to difficulties to which we shall later refer it is not possible at present to say if a small dose of typhoid toxin produces a fall or rise of temperature, the main difficulty being caused by inability to obtain an endotoxin uncontaminated by products of the artificial medium on which



NO.of animal	Weight	Approx.number of organisms		Water	Date injection
300	2335	300 million	heated	.05	28.3.12
201	1690	300 million	unheated	.05	17.4.12
731	1941	4 million	heated	48.50	24.4.12
153	2363	134 million	heated	23.60	6.5.12
OBSERVATIONS AT INTERVALS OF 30 MINUTES					
1	1800	30 million	unheated	1.0	1.7.12
2	1800	30 million	unheated	1.0	1.7.12

Chart 12.

the organism has been grown. The rise of temperature followed by fall so often seen after an injection of a large dose may be due to excess of one body or to the resultant action of a couple of which each member is pulling in opposite directions. We do not forget the depressant effects of nitrites and ptomaines from the broth. The whole question requires much further study, and cannot be pursued here. There is, however, a further fact which must be mentioned. If a massive dose of culture far beyond the lethal point be injected it sometimes happens that no disturbance of temperature is produced, though the animal may become acutely ill. This is shown in the last two experiments in Chart 12 where a culture of *B. coli* has been injected. Absence of temperature reaction after injection in distilled water of small doses above the vanishing point on the other hand we have never seen. Finally we have on several occasions noticed that if a large number of living or dead *B. typhosus* organisms deprived of all removeable



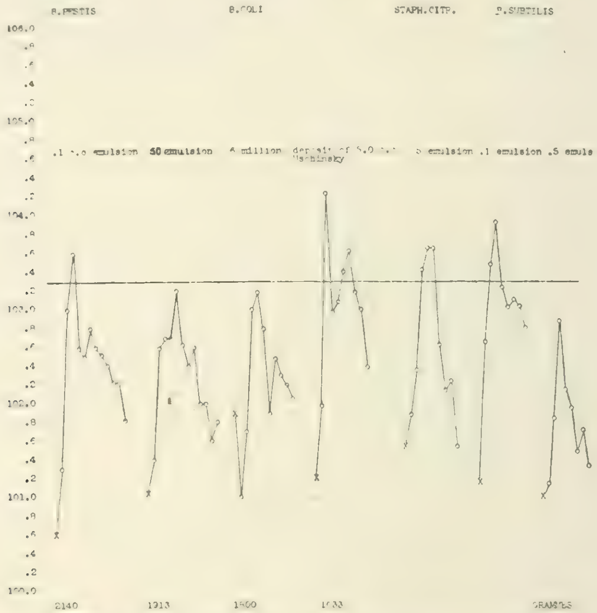
All these vaccines were centrifuged,and the deposit suspended in 1.0 c.c water except mallein [illegible].

In the case of the acne vaccine 200 million staph.aureus were mixed with the acne bacilli [illegible] alone [illegible] no fever if the heated vaccine be injected.

Interval between observations 30 minutes.

Chart 13.

traces of medium be injected in as dry a state as possible short of absolute desiccation there is not only no marked disturbance of temperature, but the animal appears to be entirely unaffected either at the time of injection or later. This is well seen in Chart 12 in the first two experiments shown. The second pair of experiments shows how the increase of diluent lowers the lethal point.



B.PESTIS

One loop of 48 hours growth on peptone agar slope was emulsified in 1.0 c.c water, and then autoclaved for 30 minutes.

B.COLI

(a) Vaccine in saline containing .5 % carbolic was centrifuged (1.0 c.c) and deposit emulsified in 1.0 c.c water. Vaccine grown on peptone agar 24 hours.

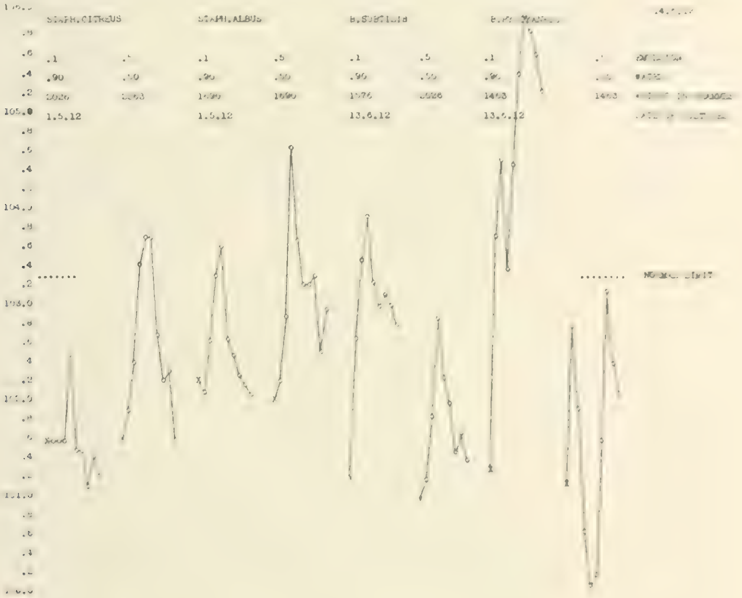
(b) Subculture from agar to Uschinsky and grown for 24 hours. 5.0 c.c Uschinsky centrifuged, and entire deposit injected in 1.0 c.c water.

14.6.12 12.6.12 29.5.12 4.6.12 DATE

Interval between observations 30 minutes.

Chart 14.

Bearing all these possible sources of error in mind we then examined the pyrogenetic properties of many different kinds of organisms. We find that we are able to divide the organisms we examined into two groups, according to whether they do or do not after death by heat excite fever when injected into healthy rabbits. We selected heat as our method of destruction because we wished to avoid the introduction of any extraneous poison, and because, as we had already shown, the pyrogen of distilled water was heat stable. In all cases unless otherwise stated, we submitted the cultures, or the centrifuged organisms, to heating for two minutes in a water bath brought to, and maintained at, the boiling point. We soon found that some varieties of organisms produce immediate fever whether injected alive or dead. It became necessary therefore, when studying the immediate and remote effect of dead organisms, to be absolutely certain that complete sterilization had taken place. The use of filters in the study of injection fever is quite inadmissible, as we have elsewhere shown, owing to the difficulty of obtaining filtrates free from inorganic impurities. In group A will be found the organisms that we have found to produce fever on intravenous injection, after excluding the fallacy arising from injection of toxic saline or water. They are *B. typhosus*, *B. coli*, *B. pyocyaneus*, *B. subtilis*, *B. melitensis*, *B. pestis*, *Micrococcus catarrhalis*, *S. albus* and *citreus*, *B. influenzae*, *Gonococcus*, *B. acne*, the cholera vibrio and also mallein. Examples are shown in Charts 13, 14 and 15. Group B on the other hand contains organisms that we have found to produce when injected after destruction by heat no fever whatever. They are *Staphylococcus*



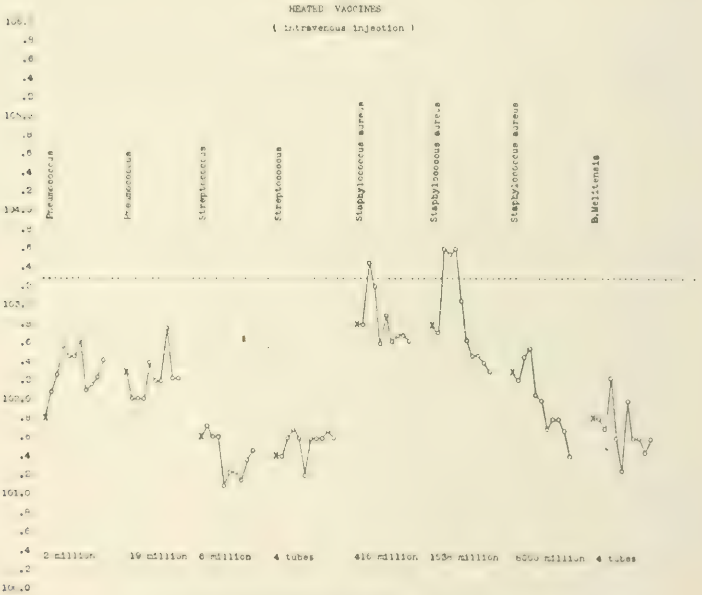
For each emulsion a [illegible] of growth was taken up in 1.[illegible] c.c of water and divided as stated.

Each emulsion was boiled for [illegible] minutes, except in the case of [illegible] which was [illegible] for [illegible] minutes.

Interval between observations 30 minutes.

Chart 15.

aureus, *Pneumococcus*, *Streptococcus*, *B. diphtheriae*, *B. anthracis*, *B. tetanus*, tubercle bacilli and also T. R. tuberculin. Examples are shown in Chart 16. In Charts 17, 18, 19 and 20 are shown the results of experiment with rising quantities of organisms in the case of many different members in group B. In no single case have we observed fever. In the case of *B. typhosus* we examined five different strains, in the case of *S. aureus* eight strains. In the case of the other organisms in groups A and B only single strains were as a rule examined. We feel therefore that the results obtained in the case of *B. typhosus* and *S. aureus* are likely to hold good for all strains of these organisms grown on artificial media. To what extent this will be found to apply to organisms where only one strain was examined can only be determined by further experiment.



Interval between observations 30 minutes.

Chart 16.

We find that the nature of the medium on which an organism such as *B. typhosus* is grown affects the pyrogenetic properties of this organism. Less fever is produced for example by growth on agar than by growth on peptone broth. We have elsewhere shown that heated organisms collected from agar plates after exposure to aerial

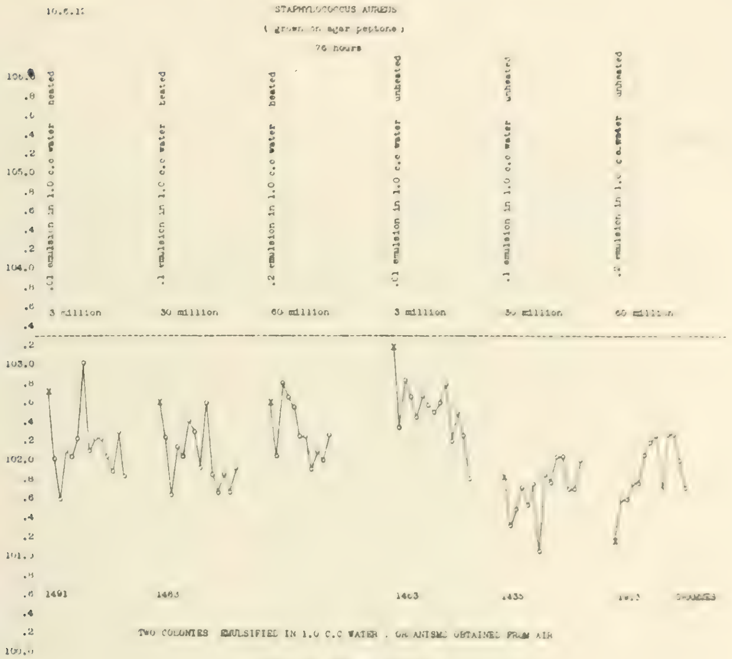
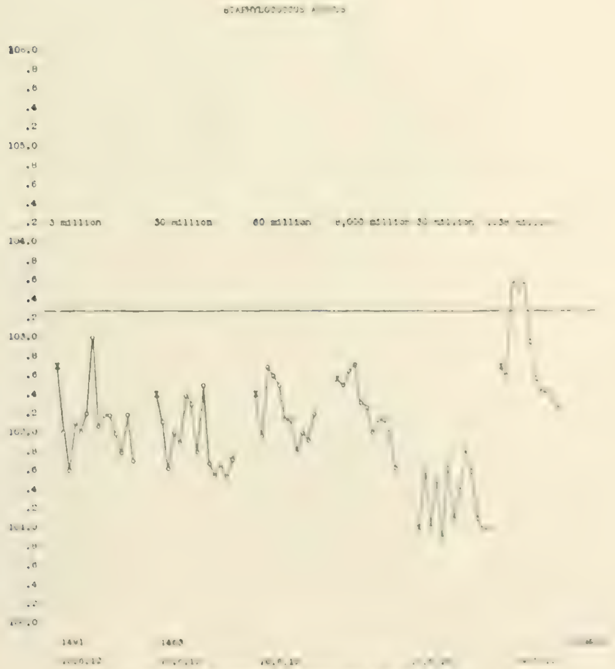
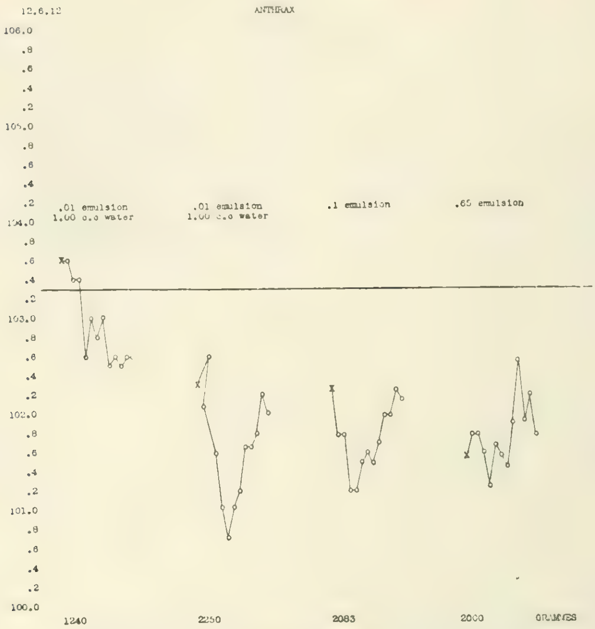


Chart 17.



Interval between observations 30 minutes

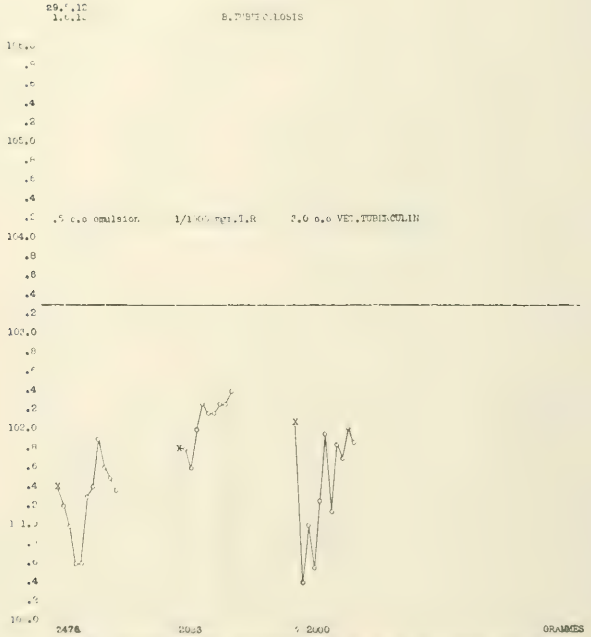
Chart 18.



One loop of 24 hours growth on peptone agar slope was emulsified in 1.0 c.c water, and then autoclaved for 30 minutes.

Interval between observations 30 minutes.

Chart 19.



In first experiment a loop of heated peptone agar culture, date 24.1.12, was emulsified in .50 c.c water and injected in 5.0 c.c water.
In second experiment tuberculin was injected in 1.20 c.c water.
In third experiment veterinary tuberculin was injected in glycerine subcutaneously

Interval between observations 30 minutes.

Chart 20.

infection may produce fever, whilst after similar treatment the same varieties of heated organisms grown in water or saline will produce none. We have also shown that this is not merely a question of numbers. We hoped that growth of *B. typhosus* or *B. coli* in non-protein media, such as those of Capaldi, or Uchinsky, might be of service in helping us to determine the source of the pyrogen in the case of organisms in group A. We found, however, that we could not obtain growth on these media of *B. typhosus*, whilst with *B. coli* that gave luxuriant growths we could never be sure that we had not carried over pyrogen derived from the broth. We find that a culture several weeks old gives rise to less fever than does a culture of 24–48 hours' growth. The type of fever produced by injections in distilled water of cultures of *B. typhosus*, an organism which illustrates very well the fever produced by other organisms in group A, after destruction by heat, is strikingly similar in every respect to the fever produced by injections of bacteria-free water or saline containing the heat stable pyrogen we have described. This is shown in Chart 21. The fever begins as a rule within a few minutes of injection, unless

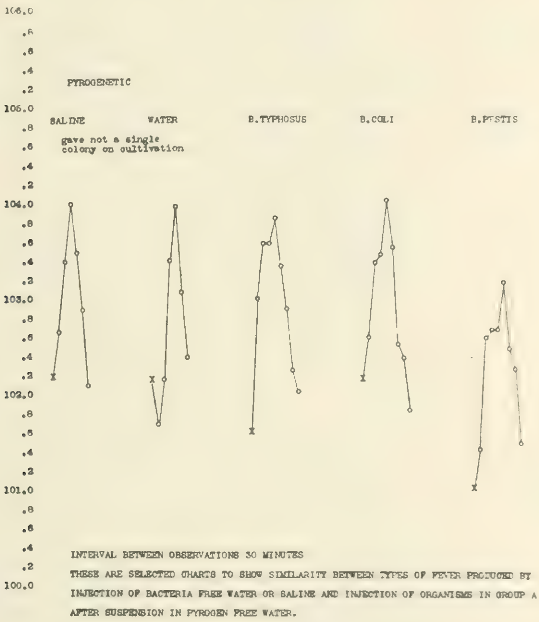
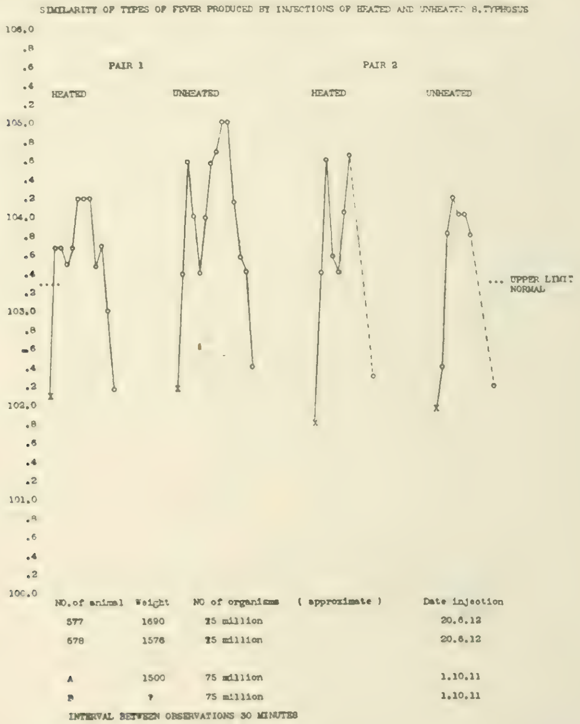


Chart 21.

too large a quantity be given, attains its maximum within 2 to 4 hours, according to the dose, and returns to normal within 5–6 hours. There is a sharp rise, a sharp fall, a short duration and no latent period. Exactly the same type of fever is produced by the injection of living cultures of *B. typhosus,* rising and falling at the same time within very close limits. Whether live or dead organisms be injected the height and duration of the fever run closely parallel if equal quantities of culture and water, relatively to body weight, are given, provided that the doses are small. This is shown in Chart 22. The type of fever curve obtained by injection of living or dead cultures in group A is quite unlike any known form of clinical fever occurring under natural conditions of infection in man or animals. We wish to emphasize the fact that though the pyrogen injected in the case of the heated culture is heat stable the fever induced by injection of the living culture lasts no longer, and does not pass into a continuous fever, as one would expect it

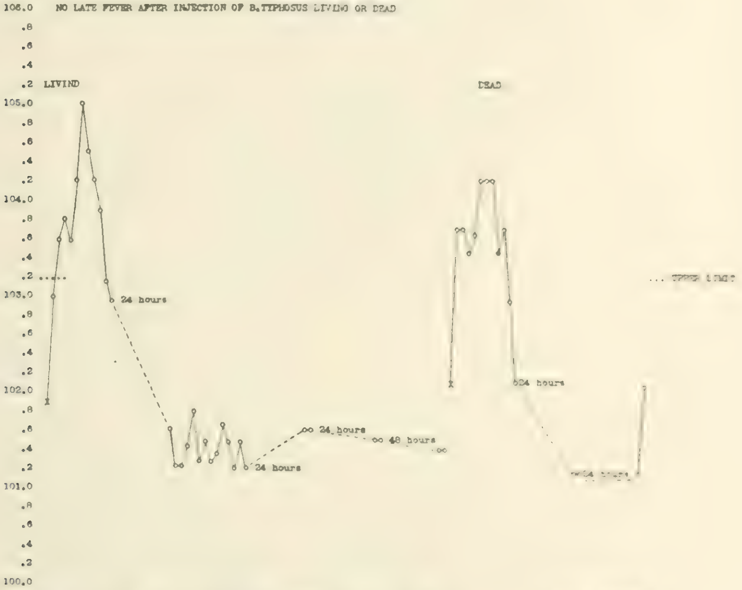


NO.of animal	Weight	NO of organisms (approximate)	Date injection
577	1690	75 million	20.6.12
578	1576	75 million	20.6.12
A	1500	75 million	1.10.11
B	?	75 million	1.10.11

INTERVAL BETWEEN OBSERVATIONS 30 MINUTES

Chart 22.

to do if a constant supply of dead organisms was being kept up owing to multiplication, and subsequent partial destruction, in the body. The pyrogen, in short, introduced with the living organism is also heat stable. The significance of this point we shall again refer to. Repeated observation for several days after injection reveals no secondary rise of temperature whether the organisms be injected alive or dead. This is shown in Chart 23. The inability of living *B. typhosus* to produce a late continuous fever in the rabbit is possibly associated with the difficulty of producing a continuous infection in this animal with this organism. This point is of particular interest in connection with the well-known power of the rabbit to act as a carrier of *B. typhosus* without the induction of symptoms of intoxication. We have found it quite impossible to produce any sign of immunity to the injection of the pyrogen of *B. typhosus*. We find it therefore difficult to believe that the typhoid carrying rabbit does not suffer from fever in consequence of being provided with an antipyrogen.



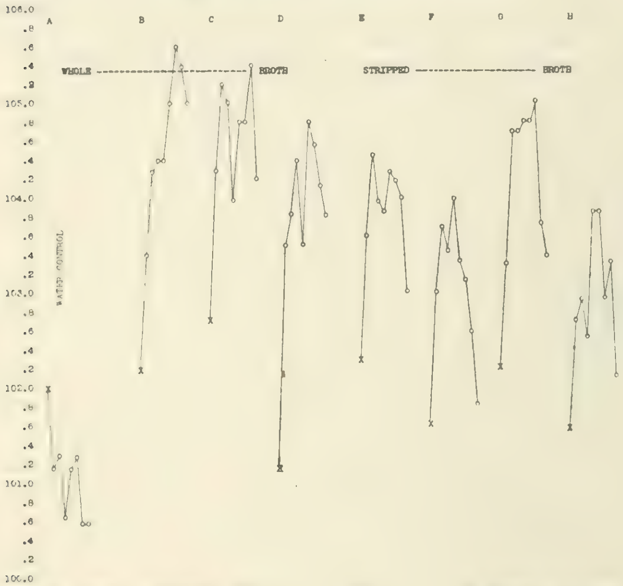
NO of animal	Weight	NO. of organisms (approximate)	Diluent used	Date of injection
568	1800	75 million	SALINE	19.6.12
577	1690	75 million	Saline	[illegible]

OBSERVATIONS EVERY 30 MINUTES EXCEPT AS STATED

Chart 23.

In order to differentiate between the immediate fever and the late fever (if any) produced by injection of living organisms in group A it is necessary to select a type that will give a continuous infection. This we are now studying in the case of *B. coli.* So far we have been unable to produce any late fever with *B. coli,* whether injected alive or dead. The next fact we wish to present in connection with *B. typhosus* is that whether we inject in suitable quantity, the culture medium plus organisms, washed organisms separated from the medium by the use of the centrifuge, or the supernatant layers of the broth from which the organisms have been thrown down, the result is always the same—fever. This is shown in Charts 24 and 25. Control observations on the apyrogenetic effect of uninfected broth are seen in Chart 26.

WHOLE HEATED TYPHOID BROTH AND STRIPPED HEATED TYPHOID BROTH



	NO.	Weight	B.T per kilo	Actual no.	Water	Date injection	Whole broth	Stripped broth
A	426	1576	nil	nil	7.80 1/202	24.5.12	nil	nil
B	418	1576	.8 m	1.5 m	7.80 1/202	24.5.12	.1 1 in 10	nil
C	420	1576	.8 m	1.5 m	7.80 1/202	24.5.12	.1 1 in 10	nil
D	421	1576	.8 m	1.5 m	7.80 1/202	24.5.12	.1 1 in 10	nil
E	422	1576	nil	nil	7.80 1/202	24.5.12	nil	.1 1 in 10
F	424	1576	nil	nil	7.80 1/202	24.5.12	nil	.1 1 in 10
G	423	1576	nil	nil	7.80 1/202	24.5.12	nil	.1 1 in 10
H	425	1576	nil	nil	7.80 1/202	24.5.12	nil	.1 1 in 10

INTERVAL BETWEEN OBSERVATIONS 30 MINUTES

Chart 24.

Moreover whether the organisms, the suspension of organisms in the broth, or in the broth deprived of its organisms, be submitted to heat or not, fever always follows if a correct dose be given. The pyrogen of each fraction is heat stable, and the fever produced in each case is of precisely the same type. The comparatively narrow limits of temperature reaction in response to the injection of pyrogen in the rabbit makes it difficult to determine if the injection of broth plus organisms gives rise to more fever than does the injection of organisms alone or of broth alone. The fact, however, that after prolonged heating to 120° C. the culture does not produce a greater effect in terms of fever than is seen after injection of the living organism affects to some extent the question of the share taken by derivatives of dead bacteria in fever production. We further find that if *B. typhosus* organisms are separated by the centrifuge from the broth in which they are grown, or are collected from agar, the pyrogen cannot be wholly removed by prolonged washing with

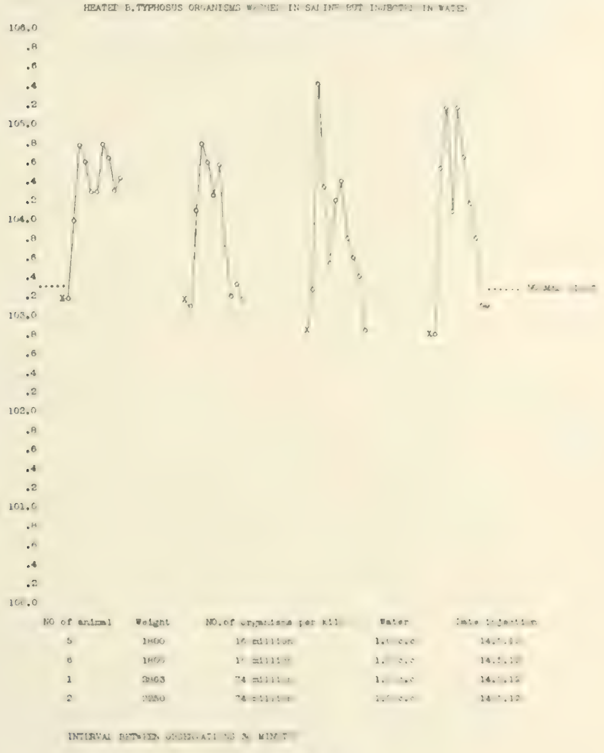
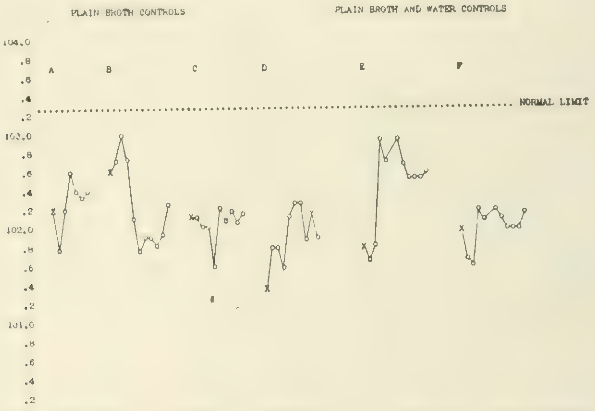


Chart 25.

water or with fresh uninoculated broth. The washings are, however, strongly pyrogenetic. If on the other hand the precipitate is washed with hydrogen peroxide, which is subsequently centrifuged or driven off by heating in the water bath, injection no longer produces fever. The same destruction of pyrogen is produced if the precipitate of organisms be freed from all excess of liquid by the aid of a Pasteur pipette, and subsequently allowed to stand for 12 hours in the cold.

After examination of numerous films we find that a precipitate of *B. typhosus* not treated in either of these ways does not stain nearly so well with methylene blue as it does if either of these methods have been applied. Moreover the morphological aspects of the stained organism itself are microscopically in no way affected by what appears to be a process of oxidation, although the degree of deglutination effected is often striking. Finally, if the organisms in as dry a condition as it is possible to get them without desiccation are submitted to prolonged heating in the water bath at boiling point the pyrogen becomes to a large extent inactive when the residue is again taken up in water. This residue loses the original colour of the medium whilst the staining properties and the integrity of the organisms as seen by the microscope are in no way impaired.

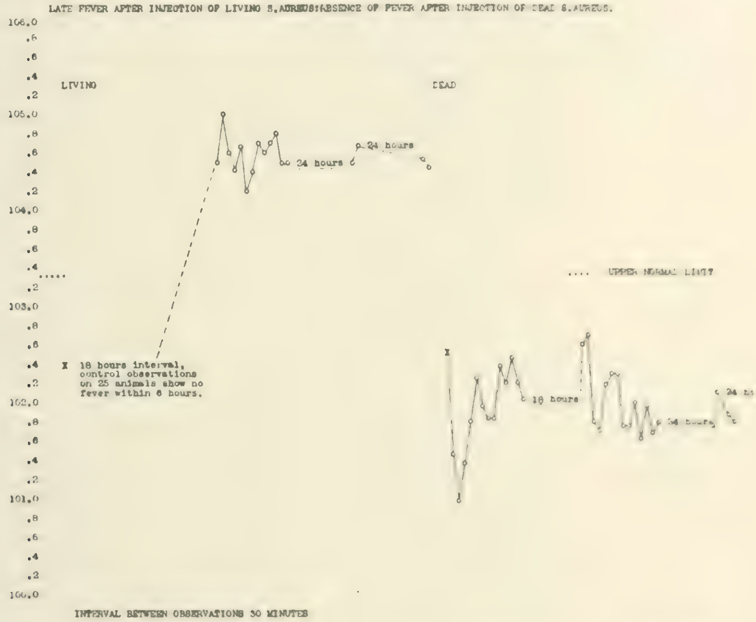


	NO. of animal	Weight	Broth in c.c		Water in c.c		Date of injection
A	49	2278	.13	1/17,523	nil		7.11.11
B	258	1913	.50	1/ 3,820	nil		25 3.12
C	332	1913	.97	1/ 1,972	nil		30. 4.12
D	334	1913	.97	1/ 1,972	nil		30. 4.12
E	330	2026	1.03	1/ 1,968	20.20	1/100	30.4.12
F	331	1913	.97	1/ 1,972	19,10	1/100	30.4.12

Interval between observations 30 minutes.

Chart 26.

If we now turn to group B a marked contrast is seen. If for example intravenous injections of vaccines of *S. aureus* are given after destruction by boiling no fever whatever follows the injection, either immediately or on any subsequent day up to a fortnight—the limit of our observations—whatever the number of organisms injected. This was shown in Charts 17 and 18. If after a small injection of a few million organisms a day, a week, or a fortnight be allowed to elapse, and if then an injection of several thousand million organisms be given, no fever ensues. If these living organisms be injected intravenously there is again no immediate fever, but on the next day fever begins, and it merges into a definite clinical fever of a well-marked type, which continues for several days. This is seen in Chart 27.



NO of animal	Weight	Quantity of culture	Heated	Date of culture and source	Date of injection
567	2590	1/10 c.c of emulsion 1 1.0 c.c water	no	[illegible].6.12 Agar	[illegible].6.12
560	2419	deposit of 3.0 c.c 16 hrs.broth culture	yes	1[illegible].6.12 Broth	[illegible]

Chart 27.

It may be well to sum up the different behaviour as regards fever production between organisms in group A, as represented by *B. typhosus*, and organisms in group B, as represented by *Staphylococcus aureus*.

The first produces, when injected alive or dead, an immediate fugitive fever, whilst the second produces when injected alive or dead no immediate fever. On the other hand, dead organisms of the first or second group produce no late fever, whilst injections of living *Staphylococcus aureus* produces well-marked fever of a continuous type, after a very definite latent period. The immediate fugitive fever with no latent period induced by injection of living or dead organisms in the first group is moreover caused by a heat stable substance indestructible by prolonged boiling or even by submission to a temperature of 120° C. in the autoclave. In this respect it behaves in an exactly similar manner to the pyrogenetic body in ordinary water or saline proved again and again often to contain no organisms capable of cultivation. On the other hand living organisms in group B are heat labile in respect of their pyrogenetic properties and produce a continuous fever after a well-marked latent period, whilst the dead organisms produce no fever immediate or remote, whether on first injection or on subsequent injection.

On the whole the evidence suggests, but does not absolutely prove, that the immediate fever which follows the injection of living or dead organisms in group A is due to introduction of an adventitious heat stable pyrogen derived from the medium, and is not due to pyrogen extracted from the organisms. If this be the case to the five fallacies we have described there must be added a sixth—the fallacy of attributing fever caused by the injection of extraneous products to derivatives of bacterial cells. The belief in the ability of dead organisms to produce fever in disease is to a large extent based on the immediate fever that follows their injection into animals, or that may follow their injection into man. Recognition of this source of fallacy, if it be a fallacy, is therefore vital.

On the other hand the fact, if it be a fact, that the immediate fever caused by organisms in group A is not a bacterial fever, is no evidence that dead organisms killed in the body are not capable of producing fever.

It is conceivable that organisms after growth on artificial media may be incapable when injected dead, of producing true fever in the body and may yet acquire this property after training and growth on the natural medium offered by an infected host.

In our search therefore for the source of the pyrogen of infective disease we have had to discard the use of organisms grown on artificial media, and to examine instead the pyrogenetic properties of organisms grown on natural media. This we have done in two ways. In a series of experiments by the first method we exposed defibrinated blood freshly drawn from the rabbit to aerial infection for 30 minutes, and then submitted the blood in a sealed vessel to a temperature of 37° C. for a period of 24 hours. This blood when injected unheated in small quantities produced a fever intermediate in point of time of appearance between the early and late fevers described. There is, in short, in these experiments no immediate fever, but a late fever with the latent period very much shortened. Control observations with uninoculated blood showed complete absence of fever immediate or remote. Cultures from the fever producing blood showed abundant growth of chromogenic cocci. Injection of the heated deposit from this blood, shown by the microscope to have a high bacterial content, produced no fever. We have therefore here evidence that the living organisms after training in fresh defibrinated blood can produce fever of a different type to that produced by organisms grown on agar or on peptone broth. In experiments using the second method we injected into healthy rabbits *S. aureus* obtained from aerial infection of agar. The organisms were then injected into rabbits and were recovered in 24 hours by centrifuging the heart's blood. In all cases injection of the killed organisms obtained from the heart-blood, produced no fever. whilst injection of sufficient quantities of the living organism recovered in the same way never failed to do so. Although we have several charts showing the points mentioned we prefer to reserve them till this branch of the work is completed. It requires in fact much further study which will be reported on in due course. Enough, however, has been said to show that it opens up a promising line of enquiry.

In conclusion we wish to glance at the practical bearing of our experiments on vaccine injections in man. If, as we believe, we have produced evidence that the immediate fever set up by injection of organisms in group A is due to the injection of highly toxic bodies of non-bacterial origin their effect for good or evil cannot be ignored. If it is an advantage in the treatment of an established infection to occasion fever by the injection of impure vaccines, standardization of the pyrogenetic content of a vaccine will in the future be no less necessary than that of its bacterial content. We have already shown that vaccines made from the same variety of organism in group A will vary from one another in respect of pyrogen unless precautions are taken to ensure the same period of growth on the same kind of medium. It is impossible to forget that the organisms for which the greatest measure of success in vaccine treatment has been claimed are those in which the content of extraneous pyrogen is highest—the acne bacillus, *B. typhosus*, *B. coli*, the *Gonococcus* and *Micrococcus catarrhalis*. On the other hand, though in the absence of further evidence it would be absurd to press the point, the vaccines which have so far given the poorest results in the production of active immunity in man are just those in which we have found no adventitious pyrogen—*Pneumococcus*, *Streptococcus*, *Staphylococcus aureus* and diphtheria. If on the other hand the presence of non-bacterial pyrogen is no merit in a vaccine the whole system of preparation of vaccines will have to be recast and steps taken to ensure destruction of pyrogen that will not involve impairment of any specific antigenic values the purified vaccines may possess. That these adventitious fever bodies have no effect on opsonic readings or other immunity reactions will also have to be shown if the hope of placing therapeutic inoculation on a sound experimental basis is to be realised. Incidentally will be noted the bearing of these experiments on the question of the relative merits of living and dead organisms as effective immunizing agents.

Finally, we feel justified in insisting that no injection experiments dealing with so-called protein fever, endotoxin fever, anaphylactic fever or bacterial fever can stand unless based on clear recognition of the numerous sources of fallacy we have named.

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FURTHER STUDIES IN EXPERIMENTAL FEVER.

By E. C. Hort and W. J. Penfold.[1]

We have recently shown that in the experimental study of fever there are numerous sources of error previously undescribed. Some of these pitfalls are fairly obvious and easy to explain—after one has plumbed their depths, as we have done with unfailing precision. Others there are which at present are not easy to understand, though they are on that account none the less definite. The following two sources of error are very difficult to explain, and as we have fully dealt with them elsewhere they need not be more than named.

(1) The first is due to the fact that the degree and duration of the fever produced by injection of constant quantities of a given pyrogenetic agent depends to a considerable extent on the volume of the diluent or suspending fluid employed.

(2) The second is due to the fact that the extent of fever which may follow the injection of constant quantities of pyrogen in constant quantities of pure normal saline (relatively to body-weight) is much more irregular than is the extent of fever which may follow injection of the same quantities of the same pyrogen in the same quantities of pure water.

A third fallacy which proceeds from the use, as a medium of injection, of water or saline that has become contaminated with the heat-stable filter-passing pyrogen we described last year, is somewhat easier to explain, though the source and nature of this elusive body are still quite unknown. Its unsuspected presence seriously impairs, however, as we have shown, the value of the experimental evidence hitherto relied on in favour of various types of injection fever. At the present moment such distinct entities of fever as water fever, salt fever, salvarsan fever, lactose fever, glucose fever, saccharose fever, protein fever, nuclein fever, tissue fever, fibrin ferment fever, serum fever, blood cell fever, hæmolytic fever and anaphylactic fever stand on no sound experimental basis. We have taken each of these one by one and have so far been unable to demonstrate their existence if pyrogen-free water or saline be employed as the injection medium. To this list we are now able to add transfusion fever in the sense of the fever which may follow the injection of defibrinated blood and which Freund describes as amboceptor fever. In the experiments quoted by Schultz and Freund in favour of this transfusion fever no controls were quoted to show (*a*) that infection of the blood had not taken place *in vitro*, (*b*) that if the blood were diluted the diluent employed was pyrogen-free. We have carried out both these controls, as Chart I will show, and we find that we cannot get fever in rabbits by intravenous injection of defibrinated blood if it is free from laboratory infection or if it has been diluted with pyrogen-free water.

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[1] From the Lister Institute. (This paper was read at the Laboratory Meeting of the Section at the Lister Institute on November 5, 1912.)

In the experiments we are about to describe these fallacies are avoided by using constant volumes of pure water as our injection medium.

Chart I.

Sterile Defibrinated Blood does not produce Immediate Fever.

Full recognition of these three sources of error is, we believe, of the first importance in the study of experimental fever. To these may possibly be added a fourth, as we showed in December of last year, and, more fully, in July of this year. We refer to it again now on account of further experiments we have carried out since July, 1912. These experiments are by no means completed, and we only bring them forward now in the hope of eliciting criticism, and, if we can get them, suggestions. This fourth fallacy, if it be a fallacy, affects the study of the fever that sometimes follows the injection in pure water or saline of laboratory-grown organisms in the dead or living state. We have already produced evidence, of a somewhat circumstantial nature, that the early fever which alone follows the injection into rabbits of certain types of living or dead organisms that have been grown on artificial media is due not to bacterial pyrogen but to a pyrogen liberated from the medium by the action of the living organisms on that medium. We also showed that certain strains of certain other types of organisms did not when injected alive or dead produce early fever, but did, if injected alive, produce late fever. We therefore grouped in a purely provisional manner the different kinds of organisms we examined according to whether they did or did not, on intravenous injection into rabbits, produce an early fever. This early fever is of short duration, has no latent period, and shows a rapid rise and a rapid fall. The fever is in every respect exactly similar to that produced by injection of water pyrogen, which, as we have repeatedly found, may bear no relation to the number of organisms capable of cultivation from the water containing it immediately prior to sterilization. On the other hand, it is quite unlike any known form of continued fever occurring in man or animals in the course of a natural infection. This is no doubt partly due to the absence of the continuous supply of pyrogen that obtains in a natural infection. On the other hand, we have as yet been unable to produce a late continuous fever in rabbits by injecting organisms in this group, no doubt on account of the insusceptibility of this animal to infection with some members of this group. Organisms, however,

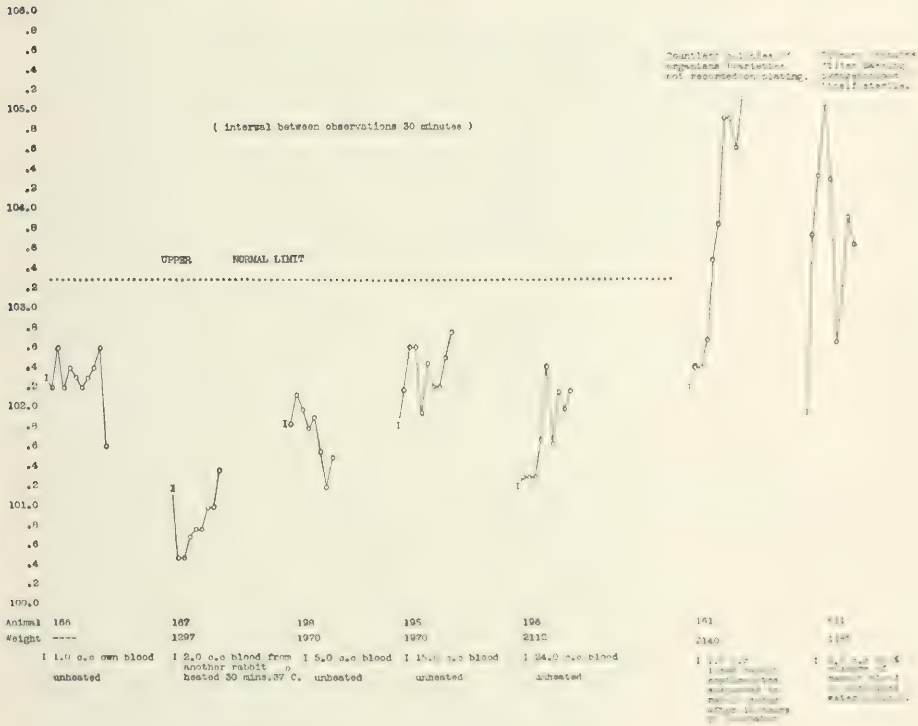

Chart I.

in the second group show, as regards certain strains, a marked contrast. Certain strains, for example, of the types of organisms that, living or dead, caused no early fever produced a late fever in the case of the living organism. This late fever has a well-marked latent period, as its name implies, a gradual rise, sometimes maintained for many days, and in cases of recovery a gradual or rapid fall. This fever was in no case fugitive, unless the animal died from an overdose, and showed all the classical features of a typical infection fever occurring naturally. Group I was well represented by *Bacillus typhosus* or *Bacillus coli*, Group II by certain strains of *Staphylococcus aureus* or of pneumococcus. In all cases the organisms were grown on the ordinary laboratory media.

Pursuing this subject still further, we now find that members of the first group, for example, *Bacillus typhosus*, preserve to a large extent the immediate pyrogenetic properties we have described in spite of age (within limits of months). We also find that certain strains of various types of organisms in Group II exhibit apyrogenetic properties (as regards early fever) both in old cultures and in young cultures of strains which had not recently passed through the animal body. On the other hand, we find that other strains of the same organisms exhibit powers of producing immediate fever in the case of young cultures but not of old. The bearing of this observation on the preparation of vaccines for therapeutic or protective purposes is, we think, worth further study. Finally we note that there are yet other strains of *Staphylococcus aureus*, for example, which produce immediate fever in the case of young cultures and of old, behaving, in fact, in this respect like *Bacillus typhosus* or *Bacillus coli*. The explanation of these differences is not yet available. It is not, we believe, a question of differences in numerical values, so far as we can tell by such quantitative experiments as we have been able to carry out. We have, on the other hand, some reason for thinking that highly important factors are the original source of the organism and the time relation of passage (followed by fresh cultivation) to injection. We have never yet failed with any strain of *Staphylococcus aureus* to obtain immediate fever if cultivation in the laboratory has been immediately preceded by passage. We require, however, much further study on all these points. In the meantime we show in Charts II and III the respective pyrogenetic powers (as regards early fever) of different strains of organisms in Group I.

CHART II.

Certain Strains of Organisms in Group II produce Immediate Fever, whether injected Alive or Dead.

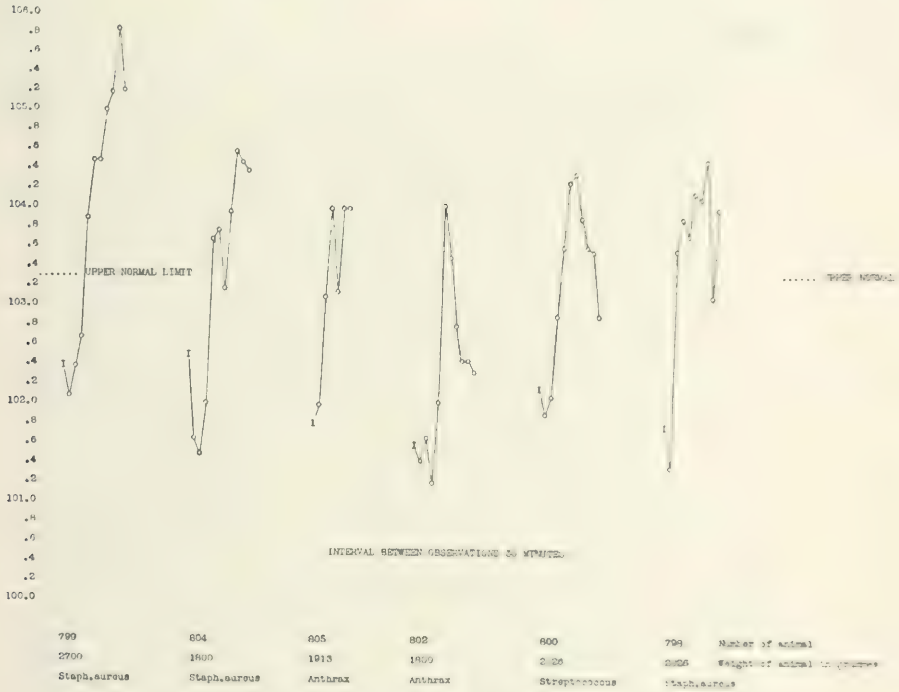

CHART II.

CHART III.

Certain Strains of Organisms in Group II produce no Immediate Fever whether injected Alive or Dead.

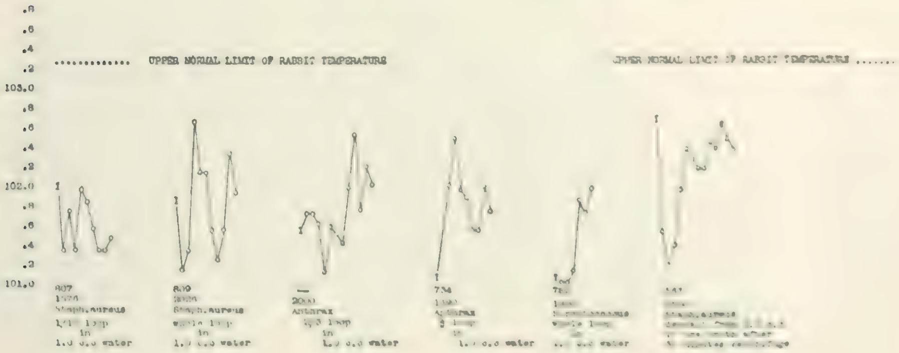

CHART III.

In further contrast to the power that certain laboratory-grown organisms have of producing immediate fever in rabbits, we show in Chart IV the effect of injection of organisms after animal passage. At first sight it appears that this chart might be explained on quantitative grounds. It is true that owing to the difficulty of recovering organisms during passage free from salts and other substances derived from the tissues we have not been so far able to devise any satisfactory method of enumeration. Precaution, however, was in all cases taken to obtain positive evidence by films that large numbers were injected. Precaution was also taken by subsequent control cultivation to demonstrate the presence of living organisms. Unless copious growth was obtained the experiments were rejected. We cannot, however, yet claim that we have positively eliminated this source of error. It is conceivable that the absence of fever is due to exhaustion of pyrogen *in vivo,* as most of the animals were suffering from fever when the organisms were recovered. We have, however, guarded ourselves against this source of error to some extent by recovering the organisms in early stages of the fever as well as in late, and by injecting as large numbers of organisms as we could secure. In all the cases shown, as well as in numerous others not charted, we have not yet been able to produce early fever whatever the stage of infection, by injecting washed organisms after passage in the living or dead state, so long as laboratory cultivation was not subsequently employed. This is true even of considerable quantities of pus recovered from young abscesses and injected at once. In some of these cases the presence of a sufficient number of virulent organisms to produce late fever, rapidly followed by death in thirty-six to forty-eight hours after injection, was clearly shown. The experiments shown in this chart strongly recall the experiments we published last year, in which we showed that we could produce no fever by injecting large numbers of dead organisms found in saline exposed to the air, provided that they were injected in pyrogen-free water. They also recall the experiments we quoted in July, in which we found that we could not produce fever by exposing defibrinated blood to aerial infection provided we injected the killed organisms, the living organisms producing marked early fever, perhaps owing to obliteration of latent period. The contrast between these experiments and the results of injecting certain organisms from nutrient media exposed to aerial infection we have already shown elsewhere. We wish, however, to repeat that further work is still required on all these points.

CHART IV.

No Immediate Fever follows Injection of Organisms after Passage.

When we turn, however, to organisms recovered after passage and then cultivated in the laboratory we find that organisms that previous to passage produced little or no immediate fever, and during passage had the same negative effect, now produce immediate fever. We see, for example, in Chart V that a certain strain of pneumococcus produced on injection no early fever. In two days after injection a continued fever set in. On the eighth day, the fever still continuing, the animal was killed. A large loopful of splenic pulp stuffed with organisms obtained under careful aseptic conditions was injected into a healthy rabbit and no immediate fever followed, but the animal died in three days. At the moment of injection an agar slope was inoculated with a fragment of splenic pulp, and by the next morning a copious growth

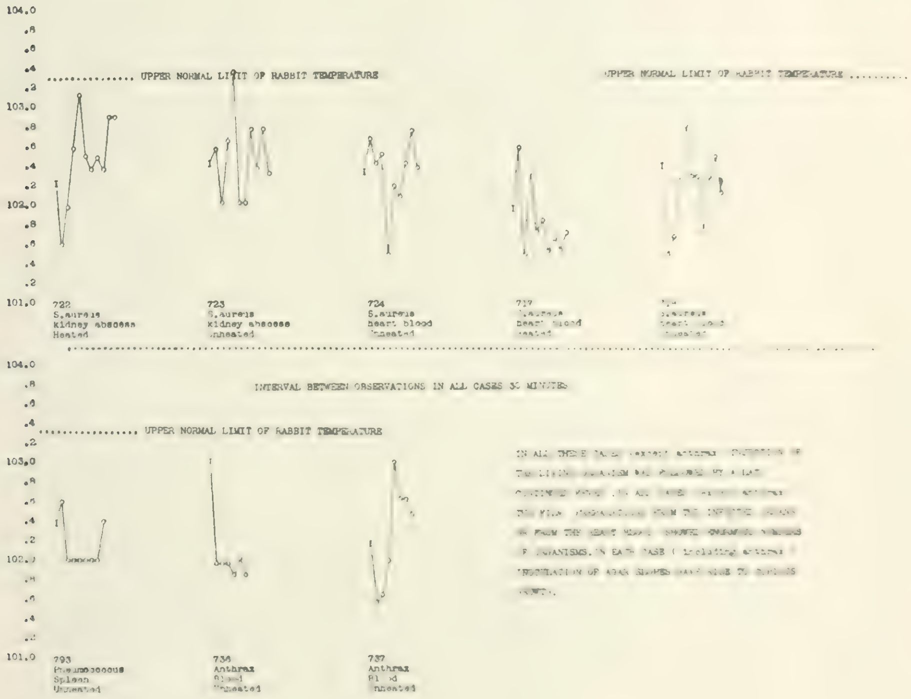

CHART IV.

was obtained. A measured quantity equal to that first injected was given to a third animal and immediate fever resulted. This animal also died. The same experiment was repeated with a subvirulent strain of anthrax, and also with a streptococcus and several strains of *Staphylococcus aureus*, recovered either from the heart blood or from kidney abscesses. An important point not shown on the chart is the fact that the immediate fever was produced by the dead organisms as well as by the living in several cases of *Staphylococcus aureus* (the only type so far tested as regards killed organisms). If this proves to be generally true, it suggests that passage plus cultivation confers on an organism not only increase of virulence demonstrable *in vivo*, but also a power with which such organism was not previously endowed, of liberating from an artificial medium a pyrogenetic substance. In other words, passage plus cultivation possibly selects from an organism that has not the power of producing immediate fever, alive or dead a strain that has that power alive and dead. We prefer, however, at present not to do more than indicate the possibilities involved in this branch of work, as further experiment is required on the effect of subcultivation without passage. If further study confirms our suspicion that the more or less immediate toxic effects quâ fever, so often seen in man after injection of dead vaccines, are partly due to contamination of such vaccines with pyrogen derived from the laboratory media, this is an accident that it may become necessary to prevent. We have already shown that living and dead *Bacillus typhosus* vaccines, for example, can be readily detoxicated, as regards immediate fever, in the test-tube by mere keeping or by oxidation, and we are now studying the relative antigenic values, in terms of test-tube reactions, of ordinary vaccines and of detoxicated vaccines. In the meanwhile we are content to submit that, in the light of our observations, the fact that certain organisms after culture in the laboratory are capable of producing immediate fever when injected dead cannot be taken as good evidence as to the source of the supply of pyrogen in infective disease.

CHART V.

The Apparent Effect on Pyrogenetic Function of Passage plus Cultivation in the Laboratory.

(Compare with Chart IV.)

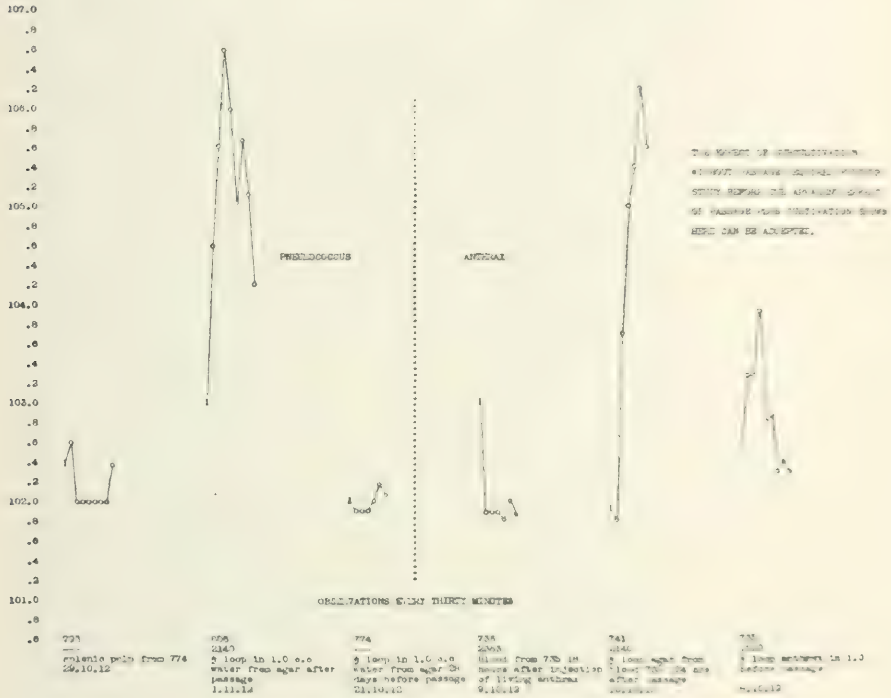

CHART V.

JOHN BALE, SONS & DANIELSSON, LTD., 83-91, Great Titchfield Street, London, W.

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A STUDY OF THE PYROGENETIC PROPERTIES OF B. TYPHOSUS.

BY E. C. HORT AND W. J. PENFOLD.
(From the Lister Institute of Preventive Medicine, London.)

To what is the fever producing effect of *B. typhosus* due? Is it due to the products of lysis or extraction of the organism, or is it due to the products of the action of the organism on the medium on which it grows? In typhoid fever in the human subject have we to deal with fever resulting from an anaphylaxis, the sensitization taking place during the period of incubation? Such are some of the questions on this subject which call for investigation. Unfortunately previous work on this subject has not given satisfactory results.

Sirotinin (1886), for example, observed the effect of intravenous injection of *B. typhosus* on the temperature of rabbits. At that time, however, the method of identification of the organism was not in a satisfactory state. And his observations on temperature were made so infrequently, the first observation being usually twenty-four hours after the injection, and in no case less than three hours, that he naturally missed the characteristic effects of injection of cultures of this organism on temperature.

Bouchard and Krehl (1895), again, described fever after injection of *B. typhosus* cultures in animals, and Krehl found falls of temperature in the case of doves, and recently Schittenhelm, Weichardt, and Hartmann have studied this subject in dogs. No one, however, of these observers took the precaution of freshly distilling the water used, or of using stored autoclaved water which had been autoclaved in sealed vessels immediately after distillation. This is absolutely essential in all experimental fever work, as we showed in 1911. We believe, therefore, that the pyrogenetic power of the *B. typhosus* has not been studied by any observer with due attention to the numerous precautions which recent experimental fever work of our own has shown to be necessary.

Our experiments with *B. typhosus* were all made on the rabbit. The injections were intravenous and the temperatures were taken in the rectum with Kew-certified thermometers. The thermometers were carefully tested from time to time.

As before mentioned, one of the first requisites of any experimental fever research is purity of reagents. The authors have shown that the injection of pure distilled water causes a fall of temperature; this is illustrated in the accompanying Chart I. This fall varies, as a rule, between 1° and 2° F. in rabbits, or more with larger injections. The injection of uninoculated nutrient broth in quantities of ½ to 1 c.cm. into rabbits of 2 kilos causes very slight rises of temperature. In quantities less than ½ c.cm. it does not produce fever even if injected in distilled water, so that the high temperature obtained by injection of small quantities of typhoid broth in distilled water must be due to either the *B. typhosus* itself or to products of its chemical action on the medium. The accompanying charts illustrate some of the points we wish to bring forward.

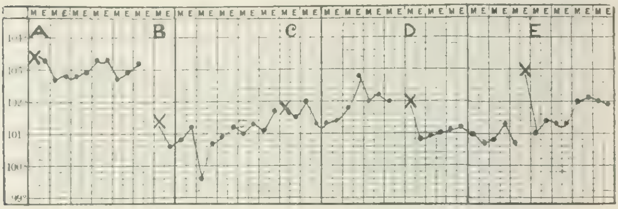


Chart 1.—Water controls.

TABLE I.

	No. of Animal.	Weight.	Water in c cm.		Date of Injection.
A	260	1,297	12.0	1/108	March 25th, 1912.
B	277	1,690	18.0	1/93	April 17th, 1912
C	273	1,406	18 0	1/78	April 16th, 1912.
D	X	2,080	52 0	1/40	(?) 1911.
E	Y	2,112	87 0	1/24	(?) 1911

Temperature Curves showing the Effects of the Injection of Rising Quantities of B. typhosus Broth in Constant Quantities of Water.

Chart 2 shows the effect on temperature of injection of mixtures of *B. typhosus* broth with distilled water. The different curves represent the temperatures of different animals. All the animals were injected on the same day, each with one two-hundredth of its body weight of distilled water to which volume rising quantities of *B. typhosus* broth were added. The mixtures were injected intravenously as stated. It will be noted that the initial temperatures were not quite the same, but, making allowances for these differences, we notice that the highest temperatures of the series are in

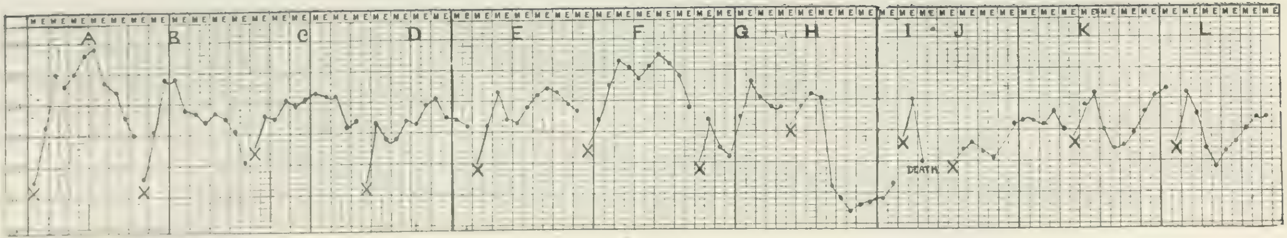


Chart 2.—Heated typhoid broth diluted (rising quantities of broth and constant quantities of water).

TABLE II.

	No. of Animal.	Weight.	Heated T.B. Broth in c cm		Water in c.cm.		Date of Injection.
A	382	1,576	0.01	1/157,600	7.88	1/200	1912. May 5th.
B	385	1,576	0 05	1/31,520	7.88	1/200	,,
C	380	1,520	0 10	1/15,200	7.60	1/200	,,
D	383	1,463	0.20	1/7,315	7.31	1/200	,,
E	381	1,800	0 40	1/4,500	9.00	1/200	,,
F	378	1,406	0.50	1/2,812	7.03	1/200	,,
G	377	1,463	0.60	1/2,438	7.31	1/200	,,
H	375	1,800	0.70	1/2,571	9.00	1/200	,,
I	400	1,800	0.80	1/2,250	9 00	1/200	,,
J	379	1,913	1.00	1/1,913	9.56	1/200	,,
K	374	2,250	1.30	1/1,730	11.25	1/200	,,
L	376	2,476	1.50	1/1,650	12.38	1/200	,,

All brindle rabbits.

animals A and B, which received the smallest dose of typhoid broth. Further, the two largest doses of typhoid broth are characterized by curious notch temperatures, neither of which attains a great height. This curious notch type of chart we have found to characterize not only *B. typhosus*, but also *B. pyocyaneus*, *B. coli*, and other organisms, and is usually, but not invariably, due to a large dose. There is, perhaps, a depressant body present in addition to the pyrogenetic, or possibly it may be the effect of a larger dose of a single body.

Rabbits H and I, which had very similar doses, showed the most profound intoxication. L died in one hour, and H had a fairly marked fall. We note that the intoxication is not proportional to dose. Our next point is to show the effect of injection of constant quantities of *B. typhosus* broth and rising quantities of water.

If a series of animals be injected with a constant quantity of typhoid broth and rising quantities of water from 5 to 30 c.cm., we find that if the dose of typhoid broth be small the increased dilution gives increased fever. If, on the other hand, the dose of typhoid broth be a little larger, with smaller quantities of water, we find that more fever results than if no water be added. The largest quantities of water may cause a profound depression, and very frequently they result in death. These latter experiments we have already communicated to the *Journal of Hygiene* (1912), and on that account we do not propose to show illustrative charts. The general result is, however, that added water greatly increases the intoxication. Further, if the dose of typhoid broth is not sufficient of itself to cause intoxication as indicated by fever, the addition of water brings it out. This is also true if the water be given before the broth into the opposite ear vein, and does not, therefore, depend on *in vitro* extraction of the *organisms*.

Our next experiments deal with the source of the pyrogenetic agent in typhoid broth.

We wished to determine, in other words, if this pyrogen of *B. typhosus* broth is derived from bacterial cytoplasm or from the broth itself. If eighteen hours *B. typhosus* broth be centrifuged for half an hour and the deposit be made up to the initial quantity of broth by means of distilled water, and if then the pyrogenetic effect of this be compared with that of the supernatant fluid, it will be found that the two are about equally pyrogenetic. We have tested this by injecting small quantities of each in doses of water equal to $\frac{1}{200}$ of the body weight of the respective animals, and we found similar fever curves to result in each case. We deduce, therefore, from these experiments that the disintegrated organisms cannot themselves be the source of the pyrogen, though this experiment does not exclude the possibility of extractives from the living organisms being the pyrogenetic agents. We have endeavoured to remove the pyrogen from the bacilli by washing with either distilled water, saline, or broth, but none of these methods are effective, though it occasionally has happened that washing has appeared to diminish the pyrogenetic power. This is, however, exceptional. We are therefore inclined to believe that the pyrogen is not a bacterial extractive. We then examined the properties of *B. typhosus* pyrogen as regards heat and oxidation.

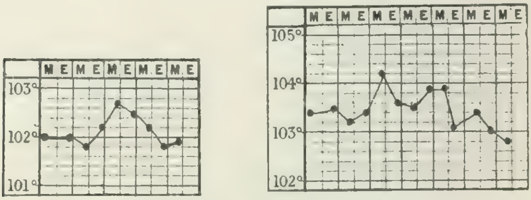
The pyrogen of the *B. typhosus* is, we find, markedly thermostable. It withstands autoclaving at 120° C. for twenty minutes. Both unheated *B. typhosus* broth and the heated gave rise to the same type of fever on injection into animals. This thermostability we also found in the case of the pyrogen of sterile water and saline which we showed to be due to a contamination capable of passing bacterial filters.

Oxidation, on the other hand, appears instantly to remove the pyrogen. If hydrogen peroxide, for example, be added to a centrifuged deposit of typhoid broth and then removed by the centrifuge and the deposit made up to its original volume with distilled water, we have found the deposit to be quite apyrogenetic. Simple standing in the cold room of the almost dry deposit has the same effect in diminishing the pyrogenetic power.

The immediate violent intoxication of *B. typhosus* as shown by high temperature, or collapse conditions with low temperature, is in sharp contrast to the results of injection of certain strains of *Staphylococcus aureus*. In the case of the latter organism the disturbances of temperature following injection of the dead organisms are negligible (see Chart 3). In the case of the living organism no effects are apparent till the next day. We have carried out a large series of experiments with different strains of these organisms, and we constantly have found a most marked contrast. The intoxication with *B. typhosus*, alive or dead, is immediate in respect of fever or collapse, whilst the intoxication produced by certain strains of *Staphylococcus aureus* never occurs with dead organisms, and is delayed in the case of the living.[1]

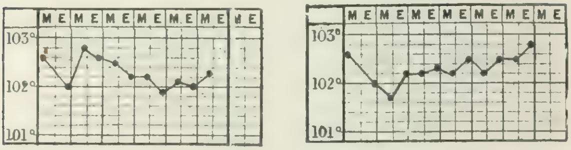
Anaphylaxis.

Friedberger and Mita (1912), in a paper on so-called anaphylactic fever, studied the effect of intravenous injection of foreign protein into guinea-pigs. They injected the protein, made up to 1 c.cm. volume in saline, into a vein in the neck which had been exposed by dissection of the animals after being fixed on an operation table.



Date of injection, June 6th, 1912. 0.2 staphylococcus, 0.8 water, boiled.

Date of injection, June 17th, 1912. 1 c.cm. staphylococcus emulsion.



Date of injection, June 10th, 1912. 2 staphylococcic, 1 c.cm. water.

Date of injection, June 17th, 1912. 1 c.cm. thick staphylococcus, 2 c.cm. water.

Chart 3.—The staphylococcus emulsion in each case consisted of one standard loop of a twenty-four hours agar slope culture in 1 c.cm. of pure water.

Friedberger and Mita distinguish the following dose of protein poisons worked with:

(1) The dose, for example, of serum just sufficient to give a fall of temperature, called the "limit of fall."

(2) The dose just smaller than the limit for fall which causes no disturbance of temperature. This they call the "upper constant limit."

(3) A still smaller dose giving a rise they call the "fever limit."

(4) The highest quantity less than that causing fever which has no effect on the animal's temperature they call the "lower constant limit."

They state that these points are so affected in anaphylactic animals that one millionth of the quantity may suffice to produce any of the respective fever limit working with some varieties of proteins. We have shown elsewhere that the guinea-pig is a very unreliable animal for temperature observations,[1] and is in fact quite useless for the purpose. In Friedberger's experience it is quite easy to determine doses of certain proteins which give the effects indicated, and the establishment of these points, he states, has enabled him to discover anaphylaxis in animals at a very early date. Now, Friedberger and Mita have shown in their paper (see Chart I) that the method they used for intravenous injection of the guinea-pig caused a fall of over 1° C., although the material injected was only 1 c.cm. of normal saline; 1 c.cm. was the standard quantity of saline with which they worked, and still they establish these various

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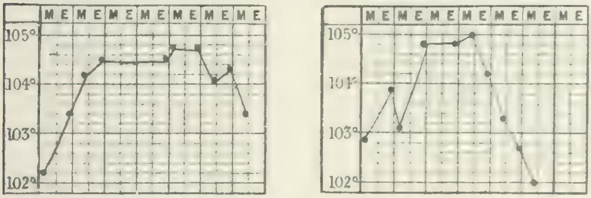
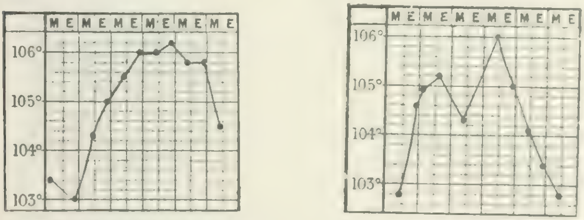
[1] *Journal of Hygiene*, October, 1912.



Date of injection, April 16th, 1912. 0.1690 c.cm. *typhosus* dilution.

Date of injection, May 3rd, 1911.



Date of injection, April 23rd, 1912. 0.49 c.cm. *Bacillus typhosus* broth.

Date of injection, May 4th, 1912.

Chart 4.

"limits" by considering movements of temperature (see curve 2 in their paper) of less than half a degree. This, of course, is fatal to their deductions. Further, if the method of operation and the saline injection give a fall of over one degree, it is difficult to see how they were able to obtain any of these limits with accuracy, and still more remarkable is it that they found no disturbance of temperature at all in so many of their animals. In uninjected animals, especially guinea-pigs, still more in animals that have had dissection of the neck done and injections given, it is practically never possible to get the straight line temperature these workers describe. We would draw attention to the fact, also, that no precautions were taken by giving control injections to ensure that the saline employed was apyrogenetic. That the apyrogenetic character of the saline must be demonstrated is admitted to be necessary now on all hands, even if the saline contains no organisms, as we have repeatedly shown. All these objections being considered, we cannot take their evidence on anaphylactic fever as satisfactory.

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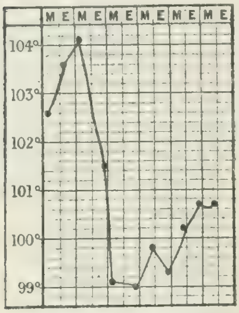
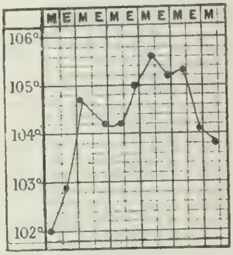
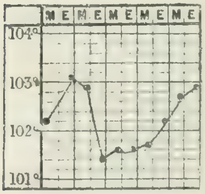
[1] Ibid.



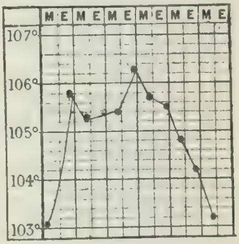
Date of injection, April 24th, 1912. 0.54 *typhosus* broth.



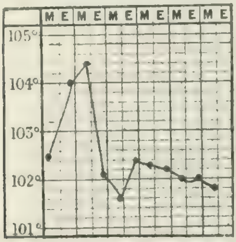
Date of injection, May 3rd, 1912.



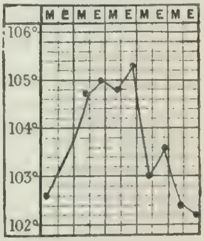
Date of injection, April 25th, 1912. 0.558 *typhosus* broth.



Date of injection, May 3rd, 1912.



Date of injection, April 18th, 1912. 0.1463 c.cm. *typhosus* dilution.



Date of injection, May 3rd, 1912.

Chart 5.

We find, as stated by Friedberger and Mita, that small doses of the poison of, in our case, *B. typhosus* give fever under conditions where large ones would give a fall. Alteration, on the other hand, of the conditions—for example, increase of the volume of diluent—completely alters the effect, as we have shown. Moreover, we frequently noticed sharp rises followed either by deep falls or by notches—that is, moderate falls and secondary rises of temperature. The regular simple temperature responses they have been able to obtain with all doses are quite unlike our experiments with *B. typhosus*. According to Friedberger and Mita, anaphylaxis to foreign protein can be demonstrated by the temperature method in spite of the fact that the stage of preparation has only lasted one day.

The accompanying charts (4 and 5) show the amounts of fever obtained in normal and prepared animals with the same dose of *B. typhosus* culture. Chart 4 shows that the fever response of the same animal to the same quantity of the same strain of *B. typhosus* was practically identical on first and second doses. It must be stated, however, that where on the first injection notch temperatures were obtained, the depressing effect was often absent on the second injection, perhaps suggesting immunity to the depressant body or effect. (See Chart 5.) We have been unable so far to obtain evidence that sensitization plays any important part in the causation of the long interval in typhoid fever between infection and onset of high fever. In short, in view of our experiments, with multiple injections of various organisms, we can at present find no justification for belief in anaphylactic fever in the sense hitherto described. The credence hitherto given to the conception has, we believe, arisen through non-recognition of the various fallacies that we have shown to be inherent to the study of experimental fever.

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Report to the Local Government Board upon the available data in regard to the value of boiled milk as a food for infants and young animals. By Janet E. Lane-Claypon, M.D., D.Sc. (Lond.).

ARTHUR NEWSHOLME,
Medical Officer,
February 24th, 1912.

(This report embodies the result of an inquiry undertaken in connection with the Board's Grant for Auxiliary Scientific Investigations.)

Part I.	Introductory.
Part II.	Experimental Evidence.
Part III.	Clinical Evidence.
Part IV.	The Special Material used and the Results obtained.
Part V.	Summary and Conclusions.

PART I.—INTRODUCTION.

There is common agreement as to the great superiority of breast milk, or of the milk of the same species, over milk obtained from any other source, as a food for infants and young animals. As will be shewn in this report, experimental evidence confirms the conclusion derived from clinical experience as to the superior results obtained by feeding infants or young animals with the breast milk of an animal of the same species, instead of with the milk of another species, and emphasises the opinion that infants should be fed on the breast unless there is an urgent reason to the contrary. There remains, however, a small minority for whom artificial feeding is necessary; these infants receive, for the most part, cows' milk in one form or another; and for them the question as to the relative nutritive value of raw and boiled milk is of great importance. The consideration of this question forms the subject of the present investigation.

This subject has assumed an important aspect, owing to the intimate connection between artificial feeding and excessive mortality among infants.

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Artificial feeding appears to have been introduced about the middle of the 18th century (32).* In the Middle Ages suckling seems to have been continued much longer than at present, its duration being considerably shortened as soon as artificial feeding became known.

Our knowledge of the degree of infantile mortality in the Middle Ages and later is necessarily very defective, owing to the incompleteness and unreliability or absence of records. There seems little doubt that the mortality among young children was extremely high.

Malthus (45) says "In London, according to former calculations, one half of the born died under three years of age; in Vienna and Stockholm under two; in Manchester under five; in Norwich under five; in Northampton under ten." He speaks frequently of the high mortality in foundling hospitals and similar institutions, and says "The child is taken under the protection of the parish, and generally dies, at least in London, within the first year."

It has been shown (57) that a high mortality among young children is in direct relationship to the mortality among infants. It is therefore highly probable that in the pre-statistical period the mortality among infants was very high. It is also probable that the prolonged suckling was as injurious to both the mothers and children of those days as it is now, and that it played a not unimportant part in the production of a high mortality among young children.

Artificial feeding spread, as was only natural, most rapidly in foundling hospitals and similar institutions; as a result, the death-rate, which was already high (cp. Malthus, quoted above), rose to an alarming extent, and regulations were enforced in regard to the breast-feeding of infants.

Towards the end of the 19th century it was discovered that milk contained large numbers and varieties of bacteria, and that the tubercle bacillus was not infrequently one of them.

The practice of boiling milk was introduced in order to destroy the bacteria. The movement started in Paris in the year 1892 by Prof. Budin (12, 13) for distributing boiled milk to artificially-fed babies spread rapidly both in France and Germany. In a large number of places boiled or sterilised milk was distributed to artificially-fed children, *under medical supervision.* The results surpassed all expectations, and it was hoped that a remedy for preventable infantile mortality had been found. These hopes have not been entirely justified. Recent researches upon infantile mortality have shown that many factors are concerned in the causation of a high infant death-rate. The experience and knowledge gained by the modern schools of pediatricians have shown that regulation of the food, and other points apparently insignificant, are often of great importance.

When it was found that the giving of boiled milk did not alone prevent excessive infantile mortality, the question arose as to whether some vital properties of the milk were destroyed by heating. Much work has been done in investigating this point, as will be seen from the following pages of this report. It is to be regretted that the problem has not infrequently been obscured by expression of opinions not based on carefully ascertained facts. In this report all known facts will be considered which bear on the general effects produced by feeding young animals upon either raw or boiled milk. This report does not deal with the experimental evidence as to the digestibility of raw and boiled milk as tested in the laboratory, the effects produced in milk by boiling, by boiling it for prolonged periods, or by heating it to a temperature above the boiling point of milk.

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* The small figures in brackets refer to the bibliography on pp. 57-60.

Confusion has been caused by the lax use of the term "sterilised milk." "Sterilised milk" in the true sense of the word is difficult to obtain; milk which has been heated to 100° C. is not sterilised, and even considerably higher temperatures are not sufficient to kill the spores which are almost always present (31).

In the literature of the subject, milk which has been brought to the boiling point of water is often described as "sterilised milk." This is a misnomer—it should be described as "boiled."

Investigation of the problem.

The milk given to a child or young animal may be the milk of its own species, or of another or foreign species. The problem can therefore be dealt with under two main divisions:

(1) The comparative nutritive value of raw and boiled milk of the same species.

(2) The comparative nutritive value of raw and boiled milk of a foreign species.

Both these divisions of the subject will be influenced by subsidiary factors, such as the age of the young animal or child, the quantity and quality of the milk given, and the environment or social condition.

Division (1) is chiefly applicable to cattle and will be dealt with in Part II. (see p. 4). It has also a limited applicability to the human species; this is dealt with fully in Part III. (see p. 15 et seq.).

Experiments have been carried out upon animals for the elucidation of the problem enunciated in Division (2), and will be discussed fully in Part II. (see p. 7 et seq.). The most important aspect of Division (2) concerns the artificial feeding of infants with raw or boiled cows' milk. The clinical evidence upon this matter is dealt with in Part III. and further clinical data, studied expressly for the purpose of this report, are considered fully in Part IV.

The clinical requirements for the elucidation of the problem appear to be

(1) A large number of healthy babies under medical supervision, and of known, and so far as possible, the same social environment, who shall have been fed for prolonged periods upon raw or boiled cows' milk.

Further there should be a control of babies drawn from the same average population fed in different ways.

Infant Consultations alone provide material of this type. Here the babies are approximately healthy, sick babies being referred to the hospital; they are under medical supervision, notes and records being kept; this means that the infant's food is regulated, and its general conditions noted. Further the babies are visited in their own homes by the health visitors of the Consultation, so that the social environment is known.

Application was made to the large Infant Consultations of this country, but although very friendly replies were received from the heads of the Consultations, either the numbers of babies were quite insufficient, or some other essential data were lacking.

Only in New York is raw milk supplied in any large quantities, all the large Consultations in Europe supplying boiled milk or ordering the milk to be given boiled.

The hope of procuring a series of babies fed upon raw cows' milk had to be abandoned, and attention was directed to the collection of evidence as to a series of babies which had been fed upon boiled cows' milk, the other conditions being also fulfilled, and to the comparison of these with another series of babies representing the average baby of the district, the last series serving as a control.

From experience gained when travelling abroad as Jenner Research Scholar, I decided to apply to the Infant Consultations in Berlin.

Professor Finkelstein, Director of the Kinderasyl, and Dr. Ballin, the Director of the Municipal Infant Consultation in the Naunyn Strasse, both most kindly and courteously placed their valuable material at my disposal.

As the material of the Consultation seemed to fulfil the requirements in some ways better than that of the Kinderasyl, I availed myself of Dr. Ballin's rich and most carefully tabulated material.

I am happy to have this opportunity of thanking both Prof. Finkelstein and Dr. Ballin most heartily for their kindness, not only in placing their material at my disposal, but also for the many offers of assistance they were good enough to make me.

The material and method of dealing with it will be considered fully in Part IV.

PART II.—EXPERIMENTAL EVIDENCE.

The experimental evidence may be grouped under the two divisions stated on p. 3.

The main evidence relating to the relative value of the raw and boiled milk of the same species is obtained from experiments upon calves.

The prevalence of tubercle among cows and its transmission to the calves by the milk has raised the question of the value of boiled cow's milk as a food for calves to one of great importance in cattle-rearing and dairy-farming.

Div. I.—*The effect of feeding calves upon boiled cows' milk.*

It has not infrequently been stated that calves do not thrive upon boiled cows' milk. The experiments which have been carried out hardly, however, support these assertions. Gerlach ([33]) found that calves fed upon boiled milk from birth did not do well after about four days. All trouble ceased, however, after the addition of Sodium Chloride, and the calves did excellently upon the boiled milk. After about a year, calves fed by this method again began to do badly, and it was ascertained that the salt had been omitted. These facts are very interesting, especially in conjunction with the experiments of Hittcher, recorded on page 6.

The experiments of Price ([56]) and of Doane and Price ([24]) in America certainly point to there being a disadvantage in the *sterilisation* of cows' milk. This food produced diarrhœa in some of the calves used.

Calves were also fed upon raw, boiled or pasteurised milk or sterilised milk, by Price, who found that the calves fed upon raw milk did the best. Four calves were used, and were fed for three days at a time upon the different forms of milk. Sterilised milk produced scouring in three out of the four calves. This milk was heated to 190° F. for ½-hour.

No data are given as to the age of the calf or the amount of milk given.

One of the calves was found to digest the sterilised milk as well as the other forms of milk.

H. H. Dean ([23]) carried out some work upon calves fed upon raw and pasteurised skim milk. He used four calves; two were fed for the first four weeks of the experiment upon raw, and two upon pasteurised milk. After a week's interval the feeding was reversed, and those which had received raw milk now received pasteurised milk and *vice versâ*. The calves also received other food. The weights of the calves and the weight of food taken were recorded. Dean concluded that no difference between the two kinds of feeding could be detected, and recommended the pasteurising of skim milk before it is given to calves.

A successful campaign has been waged in Denmark against tuberculosis, under the auspices of Prof. Bang ([2]) of Copenhagen, by the simple process of boiling the milk of tuberculous cows before giving it to the calves. Unfortunately no data as to the weight or rate of growth of the calves during the course of the feeding with boiled milk are available. I am informed by Prof. Bang that the method has now been applied in many hundreds of cases, and has been found to be entirely satisfactory. The calves are taken away from the tuberculous mother at birth, and are fed for two days upon raw cows' milk, after which they receive the milk of their tuberculous mother, either boiled or raised to a temperature of 80° C.

Hittcher ([37]) has carried out some valuable work upon the value of boiled and raw cows' milk as a food for calves, with and without the addition of certain salts. He first ascertained which salts would restore to boiled milk the property of clotting with rennet. He then performed a series of experiments in the course of which 72 calves were fed upon raw and boiled milk, to which in many cases one of these salts had been added. Full data are given, and the results tabulated. The table shows not only the weights of the calves, but also the solids contained in the milk taken, and the amount of milk taken by the calves of each series, in order to put on 1 kilo. of body-weight.

Some of the experiments were carried out over a period of 10 weeks and others over a period of 15 weeks.

The age of the calf is shown to be of great importance in dealing with the nutritive value of the food. Thus a group of four calves which received raw milk and chalk required 9·01 kilos. of milk in order to put on 1 kilo. of body-weight in the first five weeks of life, and required 14·99 kilos. of milk for the same increase in body-weight on the second five weeks of life.

If these two periods are taken together, then 12·08 kilos. of milk are required for 1 kilo. increase of body-weight.

Another set of six calves which were fed upon *boiled* milk and chalk required 13·23 kilos. of milk during the first period of five weeks and 13·25 kilos. for the second period, in order to add 1 kilo. to their body-weight.

The net results of both periods shows a slight difference in favour of the raw milk, but Hittcher justly points out how great the error might have been in drawing deductions from either period taken alone.

The table given by Hittcher, which is here reproduced in full, shows that the addition of salts is a more important factor than the actual boiling of the milk. The kilos. of milk are calculated from the values of total solids obtained by actual analysis, on the basis of milk containing 11·5 per cent. of solids.

Table of results obtained by Hittcher.

Calves.		Food given.	Kilos of milk required to produce 1 kilo. of body-weight.	Daily increase		Daily food in per cent. of body-weight (in kilograms).
Group No.	No. used.			In grammes.	Per cent.	
9	3	Boiled milk and $Ca_3(PO_4)_2$	8·87	775	161·4	1·36
10	3	Raw milk and Formalin ...	10·16	702	126·6	1·32
8	3	Boiled milk and $Ca_2H_2(PO_4)_2$	10·21	687	142·5	1·45
7	5	Raw milk and NaCl ...	10·21	866	119·3	1·315
3	6	Boiled milk and NaCl ...	10·45	803	120·1	1·317
2	7	Boiled milk alone	10·82	790	119·5	1·38
1	6	Raw milk alone	11·11	798·5	127·6	1·45
5	6	Boiled milk and Ca Citrate	11·66	697	110·1	1·38
12	4	Raw milk and $CaCO_3$ ...	12·08	863	182·3	1·93
6	6	Boiled milk and $CaH_4(PO_4)_2$	12·17	675	103·6	1·40
4	6	Boiled milk and $CaCl_2$ ...	12·59	644	107·1	1·448
11	6	Boiled milk and $CaCO_3$ ...	13·13	876	151·7	1·92

Hittcher's table cannot be summarised; it must be studied for itself in detail. He has arranged it so that there is an ascending series of figures under the heading of the amount of milk required to produce one kilogram of increase of body-weight.

If this be taken as the main criterion of the nutritive value of any food-stuff then the striking fact appears that the addition of salts is of much greater importance than the question as to whether the milk is given raw or boiled. It is difficult to estimate the value of the figures in the other columns in comparing the results of each series with one another. If in Groups 1 and 2 the amount of milk required to produce one kilogram increase in body-weight is considered, it is then seen that less of the boiled milk is required than of the raw milk; that is, the boiled milk would appear to be more nutritious.

Owing, however, to the paucity of data dealing with the amount of milk required to give an increase of one kilogram of body-weight, it has been necessary throughout this report to take the gain in absolute weight, as the criterion of nutritive value. Upon this basis, if the Groups 1 and 2, and 7 and 3, be examined, then there appears to be some advantage in favour of the raw milk over the boiled milk of the same species.

The salt-content of the milk is evidently of great importance. If the addition of salts to mother's milk can bring about such striking differences, then that difference in salt-content, which is known to exist between the milks of different species of animals, may reasonably be expected to play an important part in the nutritive value of the milk of a foreign species.

Hittcher justly emphasises the importance of conducting experiments over a long period of time, and of estimating daily the solid value of the food taken and not merely taking the weight of liquid milk given.

The work of Bang and Hittcher points to the conclusion that no serious loss of nutritive value is produced by the boiling of milk of the same species.

Div. II.—*Experimental evidence upon the nutritive value of the raw and boiled milk of a foreign species.*

There are several sources of fallacy in the work which has been carried out upon this subject, and it is difficult to decide which of them is the most important and which of them is the most commonly found.

One of these fallacies arises from neglect of the *chemical composition of the milk of the species selected for the experiment.*

Hittcher's experiments, quoted on p. 6, show the extreme value of the salt-content in considering the nutritive value of milk. The salt-content of the milks of different species is known to differ very greatly, and this must therefore affect the value of the milk for another species. Further the caloric value of the milks of different species is known to differ within very wide limits, and is presumably adapted for the needs of the particular species. Thus Pröscher (56) has shown that the chemical composition of milk varies with the normal rate of growth of the species. He estimated the rate of growth of different species by finding the time taken by that species before the initial weight at birth was doubled. On this basis he found that the rate of growth had a close relationship to the amount of protein present in the milk of the species. The quicker the rate of growth the greater the percentage of protein present.

It appears fairly evident therefore that the milk of one species cannot necessarily be considered as suitable for any other species, taken at random, and it may be supposed that the milk of a slow-growing animal will probably not have a chemical composition which will render it a suitable food for a rapidly growing species. Before endeavouring to ascertain the relative value of raw and boiled milk of a foreign species it is important to be quite sure that the milk of the foreign species selected is a suitable food for the species upon which the experiments are to be carried out. Yet, as will be seen in considering the experimental evidence in detail, these important points have been entirely ignored by some observers; while others, having discovered this after the experiments had failed, have themselves drawn attention to these points.

Another source of error is in the age of the animal.

The length of suckling varies so greatly in different animals that the state of metabolism of the young of different species at the same age after birth, cannot be considered to be necessarily at all comparable. In many of the experiments, animals have been taken after the age of suckling and therefore at an age when their metabolism is probably very different from that of animals still in the early stages of suckling. Yet these results have been taken as comparable for the study of the nutritive value of raw and boiled milk. An animal capable of utilising a mixed diet, does not give a strictly accurate comparison with an animal capable only of utilising milk.

A further source of error lies in *the very small number of animals* which have been used by many of the observers. Where only one animal for each series of experiments is used, the sources of fallacy are so great as to vitiate in a high degree the value of the experiments.

In many cases these sources of error have been recognised by the observers themselves, but only after the experiments had been carried out.

A large variety of animals have been used by different observers for the purpose of carrying out the experiments upon the subject of the nutritive value of raw and boiled milk, namely, guinea-pigs, rabbits, mice, rats, puppies, kittens, pigs, and goats. The experiments upon each species of animals will now be considered chronologically.

Experiments upon guinea-pigs.

The first experiments carried out upon guinea-pigs were those of Bolle (6). Bolle (1903) was led to carry out his experiments as the result of seeing a case of Barlow's disease in a child, which he attributed to the sterilised milk upon which the child had been fed.

This observer fed young guinea-pigs upon cows' milk which had been boiled for 5, 10, 15 minutes and longer. He found that only the guinea-pigs who were fed upon the milk which had been boiled for five minutes did well ; all the others died in periods varying inversely with the length of time for which the milk had been boiled. These results were sharply criticised by Bartenstein, who, on the publication of Bolle's results had commenced experiments upon the same lines.

Bartenstein (3) (1905) fed numerous young guinea-pigs upon raw, boiled and sterilised cows' milk. They all died. He then varied the diet, by adding to it small quantities of sterilised hay, but the animals refused the hay, and all died. Bartenstein then applied to Bolle for details in regard to the feeding. Bolle replied that the feeding had been supervised by another observer, who was no longer available for information ; that there had been a little difficulty in getting the animals to feed at first, but that it had been overcome by giving cream. No observer has confirmed Bolle's results, and not much stress can be laid upon them.

Brüning (11) (1906) fed guinea-pigs upon raw and boiled cows' milk, using breast-fed guinea-pigs as controls. Profiting by Bartenstein's experience, he allowed all the animals small quantities of hay every day. All the animals lived. The controls did best of all, next came the animal fed upon boiled cows' milk, and then the one fed upon raw cows' milk. The coats of the artificially-fed ones were not in good condition. Only one animal was used for each of the series, and the possibility of error is therefore very great.

Moro (48) (1907) fed considerable numbers of guinea-pigs upon human milk, and on cows' milk, both raw and boiled. All the guinea-pigs died in a few days, with acute symptoms suggestive of alimentary intoxication. Post-mortem examination gave no evidence as to the cause of death. Later, Moro succeeded in keeping guinea-pigs alive who were taken away from their mother immediately after birth, but were given a low vegetable diet. He then allowed the young ones to have mothers' milk for periods of 0, 1, 3, 5, 7 days, and so on. The young guinea-pigs were then isolated and given a vegetable diet. Of those taken away immediately after birth 80 per cent. died, of those left one day 30 per cent. died, of those left three days only 10 per cent. died, and of those left for longer periods *all* lived.

The weight-curves of the animals showed a progressive improvement with the length of time they were allowed to be with their mother: the superiority of the curve persisting up to the 60th day of life, when the experiment was discontinued.

These experiments show that guinea-pigs will not live upon a milk diet alone, but that they must have vegetable food. I can also personally confirm this fact, from some experiments carried out by me at the Lister Institute in 1908. (Unpublished.)

Further the period of suckling in the guinea-pig during which it receives no other form of food, is very short.

For these reasons guinea-pigs are not suitable animals upon which to carry out feeding experiments as to the nutritive value of raw and boiled milk, even apart from the question of the suitability of the milk of the particular species used. No deductions of any value can be drawn from them as to the relative value of raw or boiled cows' milk in infant feeding.

Experiments on mice.

In 1904 Keller (39) published some experiments on mice. Like those of Bartenstein on guinea-pigs, they were carried out as a result of Bolle's work, and were conducted by him personally or under his immediate supervision.

The mice were fed upon raw milk, upon milk just boiled, and upon milk which had been boiled for two hours. He found no difference between the mice of the different batches. Some digestive disturbances were present, but these were obviated by preventing the contamination of the food by fæces.

Experiments on rats.

Some experiments were carried out by myself (41) on rats, but the animals though young were no longer of an age when suckling is necessary. The rats were fed in batches of one dozen upon raw, boiled and dried milk, and a little bread. In one series those fed upon dried milk showed a slightly more rapid gain in weight than those fed upon boiled milk, and these again a rather more rapid gain than those fed upon raw milk. In two other series fed upon raw and boiled milk respectively, no difference could be detected between the rats of either series.

The sources of fallacy in deducing from these experiments any results bearing on the feeding of infants, are, that the rats were no longer of suckling age, and that they received bread as well as milk. As, however, all the batches received the same percentage of milk and bread per kilo. of body-weight, it may reasonably be inferred from these experiments that rats just past the suckling age are able to utilise dried and boiled milk fully as well as raw milk.

Experiments on rabbits.

It appears that rabbits are scarcely suitable animals for the purposes of such experiments, nor are they at all easy to use.

It is very difficult to get young rabbits to drink out of a bottle ; also rabbits' milk is much richer in fat than cows' milk and thus complications are introduced as to the amount of food required to supplement mothers' milk.

Brüning ([11]) (1906) carried out some experiments upon young rabbits, five days old. One rabbit of the litter was left with its mother, another was given raw cows' milk with the addition of cream, and the other boiled cows' milk also with the addition of cream. The rabbit fed upon raw milk died upon the eleventh day of the experiment, and the one fed upon boiled cows' milk was in a greatly inferior condition to the one left with its mother. No comparisons are possible between the effects of the raw and boiled cows' milk.

Moro ([48]) (1907) fed young rabbits upon both human and cows' milk raw and boiled. Nearly all the animals died, in spite of the fact that they took the food well. If they were allowed mixed feeding of rabbits' milk and cows' milk they rarely showed any untoward symptoms, and if these occurred the attack was warded off by stopping the cows' milk and giving only mothers' milk. The addition of cream and sugar to the cows' milk gave better results and the animals lived longer. It seems therefore that young rabbits do not thrive either on raw or boiled cows' milk or human milk, and hence are not suitable for deductions as to the relative nutritive value of raw and boiled cows' milk as a food for infants.

Experiments on dogs.

A considerable number of experiments have been carried out upon dogs, and cows' milk seems to be a suitable food for this species.

Rodet ([58]) (1896) took six puppies—four of one litter (dogs 1–4) aged from 5–6 weeks old—one of another litter of the same age (dog 5) and another rather older (dog 6).

Puppies 1 and 2 were fed upon raw cows' milk.
Puppies 3 and 4 were fed upon cows' milk, just boiled.
Puppy 5 was fed upon cows' milk subjected to prolonged boiling.
Puppy 6 was fed upon cows' milk just boiled, as for puppies 3 and 4.

As regards weight the balance was distinctly in favour of the boiled cows' milk, the increase in weight at the end of 31 days, when the experiment was terminated, being in the proportion of

638 for dogs 1 and 2,
796 for dogs 3 and 4,
276 for dog 5,
525 for dog 6.

Dogs 5 and 6 were receiving rather less milk per kilo. of body-weight than dogs 1–4.

Taking the quotient $\frac{\text{increase of weight}}{\text{daily food}}$ he found the value to be

- ·62 for dogs 1 and 2,
- ·68 for dogs 3 and 4,
- ·61 for dog 5,
- ·62 for dog 6.

Thus there was a better utilisation of the boiled cows' milk than of the raw.

Keller (39) (1904) fed two young dogs upon sterilised and boiled cows' milk respectively and found no difference between the two dogs even after three months.

Brüning (9) (1904) carried out two sets of experiments upon dogs.

In the first series he had six puppies (hounds) of which two were left at the breast, one was fed upon raw cows' milk and another upon boiled ; the two remaining puppies received raw and boiled goats' milk respectively. The puppy fed upon raw cows' milk died on the fourth day of the experiment of inhalation pneumonia, another example of the drawback of an insufficient number of animals in an experimental series.

The breast-fed puppies did incomparably better than any of the others. Then came the one fed upon boiled cows' milk, and then those fed upon boiled and raw goats' milk respectively.

The average gain for the breast-fed puppies was 113–121 grammes per day ; for the boiled cows' milk puppy 48·2 grammes per day ; for the boiled goats' milk puppy 45·7 grammes per day ; and for the raw goats' milk puppy 38·4 grammes per day.

In the second series four puppies were taken from birth. Two were fed upon the breast, the third on raw, and the fourth on boiled, cows' milk.

Here again the breast-fed puppies did incomparably better than the others.

The experiment lasted 75 days, and during this time—

- Puppy 1 on the breast had increased in weight from 165 to 2,864 grammes ;
- Puppy 2 on the breast had increased in weight from 205 to 2,215 grammes ;
- Puppy 3 on raw cows' milk increased in weight from 299 to 1,105 grammes ;
- Puppy 4 on boiled cows' milk increased in weight from 238 to 1,785 grammes.

Puppy 3 had the breast for six days and weighed 299 grammes when the raw milk was started. The figures for the increase give 2,699, 2,010, 806 and 1,547 respectively. The puppy fed upon raw cows' milk showed what appeared to be evidence of rickets and was chloroformed and examined post-mortem. No evidence of rickets could, however, be found either macro- or microscopically.

The coats of the breast-fed dogs were on the whole smoother than those of the artificially-fed dogs.

Feer's "Quotient of Increase" (26) (which is represented by $\left.\frac{\text{increase per kilo. of body-weight}}{\text{kilos. of milk taken}}\right)$ was worked out for the artificially-fed dogs of this series, and was found to be 118 for the dog fed upon raw cows' milk, and 119 for the dog fed upon boiled cows' milk.

In both the series all the surviving animals developed into very good dogs.

It appears, therefore, that cows' milk can act as a substitute for mothers' milk in dogs, and that the dogs thrive better if the milk is given boiled.

The breast-fed dogs were, however, greatly superior to the artificially-fed ones.

Moro ([18]) (1907) showed that dogs fed upon human milk remained alive but were in a very miserable condition, whereas dogs upon cows' milk did extremely well. These results taken in conjunction with the results obtained by the same observer on rabbits, show that in deducing results from experiments it is very important to use a species of animal whose milk is suitable for the other species whose growth is being investigated. The milk of one species of mammal is by no means always suitable for the young of another species. This appears again in the experiments upon kittens which will be described immediately.

Experiments on kittens.

Chamouin ([18]) (1892) took kittens 30-40 days old, and fed them on raw and boiled cows' milk, three kittens on each kind of milk. There was also a control breast-fed kitten. The experiment lasted 12 days. The control kitten put on 560 grammes in weight. The kittens fed on raw cows' milk put on 172 grammes, and the kittens fed upon boiled cows' milk put on 349 grammes each. It seems, however, that the artificially-fed kittens were not in a good state of health, and that not improbably had the experiment lasted much longer the artificially-fed kittens would have died.

From some work carried out by Grünbaum for the Local Government Board, (referred to in the Annual Report of the Medical Officer to the Board for 1906) it seems fairly evident that cows' milk is not a suitable substitute for mothers' milk for kittens, until they have reached an age when mothers' milk is no longer necessary.

Vincent ([66]) (1911) has carried out some experiments upon kittens. These experiments as such do not come within the scope of this report ; but since he deduces from them that boiled milk is harmful for babies, brief mention must be made of them.

The kittens taken were of the age of two months, and the feeding was carried out by the animal attendant. Milk was brought from the Infants' Hospital, and after the milk had been raised to 200° F. it was incubated for 24 hours at 85° F. In most of the series the milk, after heating, was inoculated with special organisms, the action of which it was desired to study.

All the kittens fed upon these foods died. It is clear that no kitten was fed upon boiled milk as ordinarily meant by the term, since even where there had been no inoculation of the milk, it was incubated for 24 hours before administration and therefore not comparable with milk which, in accordance with usual practice, is boiled and used very shortly afterwards.

None of the experiments on kittens seem to have given results upon which any reasonable deductions can be based in regard to the relative nutritive value of raw and boiled cows' milk as a food for either infants or kittens.

Experiments on pigs.

Brüning (11) carried out experiments upon young pigs.

The litter used consisted of seven pigs. Pigs A, B, C, were left with their mother, but were only allowed to feed at stated intervals, and were weighed before and after each feed, so that the total quantity of milk taken was known. Pigs D and E were fed upon boiled cows' milk, and pigs F and G upon raw milk.

The initial weights of the pigs varied from 912–1,365 grammes. The supply of mothers' milk was poor, and the breast-fed pigs obtained a much smaller quantity of milk than the artificially-fed pigs. All ate well and put on weight well.

No appreciable difference could be detected between the different pigs D and E, and F, and G, but the artificially-fed pigs doubled their weight rather sooner than the breast-fed ones ; possibly owing to the shortage of food-supply in the latter case.

Feer's quotient (increase per kilo of body-weight ÷ kilos of milk taken) gave a value of 165 for the breast-fed pigs, of 65 for the pigs fed upon boiled cows' milk, and of 60 for those fed upon raw cows' milk.

Mothers' milk is therefore very much better utilised by pigs than cows' milk, and boiled cows' milk rather better than raw.

Bamberg (1) (1910), realising that in all the experiments which had been carried out so far ordinary impure market milk had been used, undertook some experiments upon pigs, in which he used the purest milk obtainable in Berlin. The bacterial content of the milk was taken and the milk was found to be almost germ-free throughout the experiment.

The milk was obtained from the Viktoria Park Dairy.

A litter of eight pigs was taken immediately after birth.

Of these pigs—
A and B were fed upon raw cows' milk from the Viktoria Park Dairy.
C and D were fed upon boiled cows' milk from the Viktoria Park Dairy.
E was fed upon raw cows' milk (market milk).
F was fed upon boiled cows' milk (market milk).
G and H were fed upon the breast.
Pigs G and H doubled their weight on the 14th day.
,, A ,, B ,, ,, ,, ,, 17th day.
Pig F doubled its weight on the 19th day.
,, C ,, ,, ,, ,, 20th day.
,, D ,, ,, ,, ,, 23rd day.
,, E ,, ,, ,, ,, 32nd day.
Pigs C and D did not seem quite so happy as A and B.

As regards weight therefore the pigs fed upon raw germ-free milk did rather better than those fed upon the same milk boiled. But the pig fed upon boiled market milk did very considerably better than the one fed upon raw market milk.

Feer's quotient was worked out for the artificially-fed pigs and the following values were obtained :—

	Pig A.	Pig B.	Pig C.	Pig D.	Pig E.	Pig F.
1st Week ...	85·5	68·1	54·0	49·2	19·1	55·7
2nd ,, ...	61·2	68·0	60·0	41·2	29·5	30·8
3rd ,, ...	55·2	30·1	26·5	29·1	47·0	21·4
4th ,, ...	37·8	21·8	18·6	22·9	38·4	15·8
5th ,, ...	22·1	16·0	14·1	19·1	20·7	9·3
6th	16·6	9·9	8·3	12·7	13·8	9·2
7th ,, ...	13·2	7·9	6·0	8·8	7·6	5·1

As a whole therefore pigs A and B utilised their food rather better than pigs C and D. But pig F utilised its food better than pig E. That is raw germ-free milk seems to be better utilised than boiled, but boiled market milk appears to give better results than raw.

After seven weeks the pigs were returned to their owner, who reported later on that the artificially-fed pigs were all inferior to the breast-fed pigs, but that no difference could be detected between the different artificially-fed pigs.

Experiments on goats.

Brüning (9, 11) carried out two sets of experiments on goats, which are however, really supplementary the one to the other, and can be taken together. Each litter had consisted of three kids. These were fed as follows :—

First Litter—
  Kid 1. Breast-fed.
  Kid 2. Boiled mothers' milk.
  Kid 3. Boiled cows' milk.
Second Litter—
  Kid 4. Breast-fed.
  Kid 5. Breast twice a day and other feeds raw cows' milk.
  Kid 6. Raw cows' milk.

Kid 1. Doubled its weight on the 15th day. The value for Feer's quotient was 50·2.

Kid 4. Doubled its weight on the 16th day. The value for Feer's quotient was 53·1.

Kid 2. Doubled its weight on the 22nd day. The value for Feer's quotient was 28·4.

Kid 5. Doubled its weight on the 22nd day. The value for Feer's quotient was 25·0.

Kid 3. Doubled its weight on the 20th day. The value for Feer's quotient was 24·0.

Kid 6. Doubled its weight on the 25th day. The value for Feer's quotient was 21·0.

The initial weights of the animals were very different, so that some of them had put on considerably more weight than the others. Feer's quotient shows the nutritional value of the food, although it does not show the caloric value of the food taken.

These experiments bring out the great superiority of mothers' milk over that of cows' milk as a food for kids. They also show slight superiority of boiled cows' milk over raw.

Brückler (8) (1907) carried out an experiment on goats on identical lines to those of Brüning. Two litters were used, and two goats, for each method of feeding. The milk given was collected with all possible precautions, and was as far as possible germ-free. Brückler obtained a rather more rapid increase in weight with the raw cows' milk than Brüning did, but Feer's quotient was higher with the boiled cows' milk than with the raw.

Goats seem therefore to do fairly well on cows' milk, but the results are very inferior to those obtained with mothers' milk.

If cows' milk be given there is a slight advantage in giving it boiled.

Summary of results of Experiments given in Part II.

Summarising the results of preceding pages very briefly it can be inferred :—

(1) That there is no evidence to show that boiled cows' milk is markedly inferior to raw cows' milk, as a food for young calves, at any rate after the first 2 days of life. (Cp. Bang.)

(2) That the salt content is of great importance. (Cp. Hittcher and Gerlach.)

(3) That, if young animals are fed upon the milk of a suitable foreign species, they appear to thrive somewhat better if the milk is given boiled, than if given raw, the only exception being in the case of germ-free milk. (Cp. Bamberg.)

(4) That, in those cases where the health of the animals was inquired into, after the cessation of the experiment, no difference could be detected in the animals fed by different methods of artificial feeding.

(5) All the animals fed by different methods of artificial feeding were inferior to the breast-fed animals, both at the time of the experiment, and afterwards.

PART III.—CLINICAL EVIDENCE,

The amount of clinical material which is available for the purpose of this report is unfortunately rather scanty.

The only class of hospital cases which can be considered at all suitable for the investigation of the nutritive value of raw and boiled milk, are those of infants suffering from the lesser degrees of mal-nutrition. As hospitals are institutions for the reception of the sick, children in the early stages of nutritional disturbances do not as a rule gain admittance as in-patients. For this reason most of the work has been carried out upon children showing definite wasting, whilst some of them had reached the later stages of atrophy.

It is impossible to say how far the metabolism of such children is directly comparable to that of healthy children, but it is probable that any difference in the relative digestibility and nutritive value of the food given, would be more pronounced in the case of a sick than of a healthy child.

Any nutritional difference which might exist between raw and boiled milk might therefore reasonably be expected to be more pronounced in the case of clinical evidence than of the experimental evidence just studied. It will be seen, however, that different observers are not agreed upon the relative value of raw and boiled milk in the treatment of atrophic children.

It is usually difficult in hospital practice to keep the children under observation for a sufficiently long period to study to the full the effects of the observations made in the hospital. As soon as the children are considered to have recovered they are sent home and are lost for the purposes of the research. Thus, their history is only known at the time of the acute disturbance.

The data given in papers on clinical research are often insufficient and control experiments in most cases are wanting.

The details of the source and preparation of the milk given are frequently entirely omitted, nor is it by any means always clear whether the milk spoken of as "sterilised" is really boiled or sterilised, or *vice versâ*. For this reason some of the cases fed upon "sterilised" milk will be dealt with although literally outside the subject of "boiled" milk.

The quantity and quality of the milk used is frequently neglected, except in the case of metabolism experiments, where it is evidently essential that it should be known. The estimation of Feer's quotient of growth, which is justly considered of great value in the experimental evidence on animals, (see Part II) receives scant, if any, attention.

A serious source of error lies in the small number of children which it is possible to have under observation in hospital, whose condition is such as to render them comparable for experimental purposes.

Hence much of the work done must be taken as dealing rather with isolated cases, than as proving any general facts in regard to the relative nutritive values of raw and boiled milk, as a food for infants. The literature contains a large number of lectures, papers, and discussions upon the question now under consideration, but only those containing definite facts have been dealt with here. There is no doubt that many members of the medical profession both in this country and abroad are strong advocates of raw milk in private practice. I have not however met anyone who was able to give any definite data showing the superiority of raw over boiled milk as a food for infants.

In private practice where raw milk is ordered, the medical adviser selects a dairy of known reputation, and special precautions are often taken in the care of the milk. It is therefore intrinsically a better milk than that which is commonly employed when boiled milk is prescribed to the poorer classes of the population. A higher price is paid for the milk, in order to have a superior milk which can be given raw.

It is impossible to avoid the impression that many of the opinions formed by medical men in private practice, are based upon a comparison between the well-to-do baby fed upon expensive raw milk, and under proportionately favourable conditions, and the baby of the poorer classes, fed upon inferior boiled milk, and under greatly inferior social conditions. Such comparisons even if based—as they are in some cases—upon some degree of knowledge of the progress of the baby's weight are valueless. It is only by feeding babies upon raw and boiled milk of the same quality, that any reliable evidence can be obtained.

Such evidence as is available will now be considered in chronological order. The clinical evidence—like the experimental—can be divided into the two main divisions :—

(1) Those fed on raw and boiled milk of the same species, and
(2) Those fed on raw and boiled milk of a foreign species,

and will be considered under these two headings.

Div. I.—Infants fed upon boiled milk of the same species.

Under this heading come infants which have been fed upon boiled mother's milk, or boiled human milk, that is upon milk of a woman not their mother.

In this country where wet-nursing is rarely if ever practised, cases in which it might be advisable to express and boil human milk, only occur in very exceptional circumstances, and do not ordinarily come into clinical consideration. On the Continent, however, where wet-nursing is extremely common, such cases are by no means infrequent. When syphilis in a woman is suspected but not proved, it is evidently undesirable that she should suckle any child other than her own. In these circumstances the milk may be expressed and boiled, and the child fed with human milk from a bottle without fear of infection.

This has been done in many cases, and the subject has also been studied experimentally, in a few others. Opinions are not quite agreed as to the results.

Moro ([49]) (1902) describes two cases of weakly children who received first of all the breast, and were then fed upon the expressed milk, which had been boiled for 10 minutes. The children showed a markedly inferior gain in weight upon the boiled human milk, to that which they exhibited upon the breast milk.

The average gain in weight noted was as follows.—

In case I :—

In 14 days on the breast the average gain was 26½ grammes per day.

In 8 days on the bottle with raw human milk the average gain was 37 grammes per day.

In 10 days on boiled human milk the figure was 9·9 grammes per day.

In case II :—

In 7 days the average gain on the breast was 14 grammes per day.

In 5 days on raw human milk in a bottle it was 20 grammes per day.

In 7 days on boiled human milk it was 9 grammes per day.

It would be unwise to base any assertions upon two experiments but as far as the experiments go, it would appear that some of the value of the human milk is lost by boiling. It is curious that both children showed a greatly increased average gain per day, when fed upon the human milk in a bottle, and this rather suggests that they were getting more actual food in the shape of a larger quantity of milk. It might be that different results would have been obtained if the milk had not been boiled for as long a time as 10 minutes. Moro attributes the effect produced to a loss of the so-called "protective substances" of the milk.

L. F. Meyer (46) (1906) in the course of his "Exchange" experiments where three children were fed upon human whey and the protein of cows' milk, and then upon cows' milk and the protein of human milk, found that if the human whey was boiled the good results obtained with the first mode of feeding were greatly reduced. As, however, the experiment was only carried out over a very few days, and was an isolated one, it cannot be taken as proving that substances necessary for health are lost by the boiling of milk.

It would appear also that Meyer himself does not lay much stress upon this experiment since in his book (1910) in conjunction with Langstein (42) he says "clinical observation could not show any advantage of raw cows' milk over boiled, and recent experiments have also shown that the boiling of human milk does not cause any deterioration of its nutritive value."

Potpeschnig (55) (1907) carried out some work with a view to ascertaining whether these same hypothetical substances having a protective function were injured by heating to 60°C., this being the temperature to which v. Behring considered that milk might safely be heated, without undergoing any loss of nutritive value.

Two children, both premature, were fed at the breast of a wet-nurse until it was evident that they were doing well, and the weight-curve showed a daily satisfactory increase. This was for a period of three days. The children then received the milk after it had been expressed and kept cool: all aseptic precautions being taken. Both children showed a transient loss of weight, but in a few days the weight curve again began to rise, and showed a steady increase. This period lasted five days.

The expressed milk was now heated to 60°C. for ½ hour, and was then stored in the cold room for use as required. The period of this form of feeding lasted 17 days, and the weight-curve showed a steady rise throughout the entire period, nor could a disturbance of any kind be detected. After this, the wet-nurses being no longer available, the children received boiled cows' milk, and the weight-curves continued to rise just as steadily as with the boiled human milk.

The author considers that these children are no criterion since they did as well upon boiled cows' milk, as upon human milk, and thus he says were evidently capable of manufacturing their own "protective substances."

E. Müller (50) (1908) carried out a very interesting experiment upon a child under his care. This baby was premature and weakly and was 25 days old when it came under his treatment. The child was first of all put upon the breast of a wet nurse, and made very little progress, the average gain in weight being 12 grammes per day. The baby was therefore put upon a diet of raw human whey and raw cows' fat and casein; this feeding was continued for five days, during which time the child gained weight at the rate of 44 grammes per day. The author considered this increased gain in weight due to the increase of protein material in the food. Next it received cows' whey and human fat and casein both raw, for 14 days, but the results obtained were not similar throughout the period. For the first five days the child made good progress and put on weight at the rate of 33 grammes per day, but during the last nine days it only gained 30 grammes in the whole period. There was also a tendency to diarrhœa. It was then put back upon human whey and cows' casein and fat, and again made good progress, gaining 17 grammes per day for three days. The human whey was now boiled and mixed with the cows' casein and fat, and the child gained in weight at the rate of 15 grammes per day for 33 days. It would seem therefore that no harm was done to the nutritive properties of the human whey by the boiling of it.

Muller points out that the upholders of the theory of "protective substances" in milk, all agree that these are present in the whey; hence this case in which he fed a weakly child with success upon boiled whey (in which presumably these same substances, if present, were destroyed) is of considerable interest.

Finkelstein ([28]) mentions that he fed six babies upon boiled mother's milk, and found that they did not do so well as upon the raw human milk, and concludes therefore that the milk of the same species loses some of its value by boiling; in contradistinction (as will be seen below, p. 22), to that of a foreign species. He does not give any data upon this point.

The most numerous cases I have been able to discover are those of Prof. Thiemich of Magdeburg. They are unfortunately as yet unpublished, although the data are available, but Prof. Thiemich has been so kind as to give permission to me to quote his experiences for the purpose of this report. He has furnished me with a short report of his results, as follows:—"In my wards the milk of all the "new wet-nurses is given boiled only, until Wasserman's and Stern's "reactions* have been carried out."

"If it happens, as it frequently does, that one or other of the "reactions is positive or doubtful, and there is no detectable evidence "of specific trouble in either mother or child, I sometimes keep the "wet-nurse for many months, and during the whole of this period her "milk is only given boiled. I may add that in the case of an infant "living with its parents which requires a wet-nurse or human milk "for "allaitement mixte" it is only in very exceptional cases that I "allow actual suckling. Otherwise only expressed and boiled milk, "human milk, is given by the bottle in suitable quantities. Similarly "the milk of a feverish wet-nurse is boiled, not because of the risk of "the direct passage of any organisms in the milk, but because of the "possibility of outside contamination of the milk.

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* Stern's reaction is a modification of Wassermann's and apparently sometimes clears up a doubtful diagnosis obtained by Wassermann's method.

"On this system I have now seen a great number of children "improve just as well upon boiled human milk, as happens with raw "human milk.

"In a smaller number of cases raw and boiled human milk have "been given alternately and systematically for various periods of "days and weeks with the same result, namely that no difference "could be detected."

It is much to be hoped that Professor Thiemich will shortly publish his data in full, so that the actual figures on this very interesting point may be available.

The data available are evidently insufficient to enable any definite conclusion to be drawn in regard to the comparative nutritive values of raw and boiled human milk. It will be realised, however, that this is an entirely different problem from that of the comparative nutritive values of the raw and boiled milk of a foreign species. The great variety in chemical composition in the milks of different species, leads to the assumption that the milk of each is adapted especially for the needs of that particular organism. It is conceivable therefore that in such a complex and presumably nicely-adjusted food as milk, the slight changes produced by boiling might have a more important effect upon the nutrition of the young animal of the same species, than would be produced by the corresponding changes induced in the less-well adjusted milk of a foreign species. From the evidence above given there can be no doubt that many babies thrive well upon boiled human milk, and hence it must be supposed either that the changes produced by boiling are insignificant, or that the milk is not so finely adjusted to the needs of the particular species as to lose by boiling any appreciable portion of its nutritive value. The importance of salts in the feeding of calves upon raw and boiled cows' milk has already been dwelt upon (pp. 4, *et seq.*).

Div. II.—Clinical Evidence in regard to Infants fed upon Raw and Boiled Milk of a Foreign Species.

As has been already pointed out, most of the papers which have been published deal with children in different stages of atrophy. In this report only such evidence as treats of the feeding of these with raw and boiled milk has been considered, all other treatment of atrophic infants being neglected.

In 1891 Uhlig (^{62}) quoted 39 cases of children suffering from various degrees of mal-nutrition, who all did well on sterilised milk. No control cases were taken.

In the same year Leeds and Davis (^{22}) published an account of the results of feeding sick children—all apparently suffering from diarrhœa—on sterilised milk. The results were not good, some of the children dying from apparent starvation. No improvement followed the peptonising of the milk, although Fowler's solution and cod liver oil helped. Marked improvement occurred in such children as received a few breast-feeds in the day, together with the sterilised milk. No controls seem to have been carried out with raw or pasteurised milk.

Much stress cannot be laid upon these results, since there were no controls, although Davis states that he has seen children who were not improving on sterilised milk do well upon raw. The milk in these cases of Davis's above quoted was sterilised for half an hour.

Variot (63) (1898) at a meeting in Paris quoted 30 cases of atrophic children who had attended his consultation at Belleville, and who had all done very well when fed upon sterilised milk. He gave no control cases.

Palmer (52) (1900) described the satisfactory results which he had obtained in America with raw milk, and advocated its use. He gives no control cases fed upon boiled milk, and, moreover, had the great advantage of the large ice-supply of American cities. No actual data are given.

Monrad (47) (1902) published six cases of atrophic and dyspeptic children who had improved when fed upon raw milk. He considers that only certain cases need raw milk. No controls fed upon boiled milk are given.

Czerny (21)(1902) at the Breslauer Klinik fed atrophic children upon both raw and boiled goats' milk, the goats being kept upon the premises. He was unable to detect any appreciable difference between the children fed upon raw and boiled goats' milk; both sets were very constipated, those fed upon raw milk rather more so than those fed upon boiled milk. The improvement in all cases was very inferior to that which usually occurs when similar cases receive mother's milk.

Halipré (36) (1904) quotes the case of a child whose weight was increasing at the rate of 7 grammes per day while fed upon sterilized milk. When given raw milk the increase rose to a rate of 30 grammes per day. He states that he has seen other similar cases. The degree of sterilization of the milk is not given.

Hohlfield (38) (1905) published eight cases of children suffering from various stages of mal-nutrition who were fed for the most part upon raw milk, some however received raw milk alternately with boiled milk over periods of about one month. All the children were ill, some of them severely. Three had only raw milk, two vomited boiled milk, and another put on more weight on raw milk than on boiled milk. The remaining two were twins, of whom the sicklier child was put upon raw milk, and the healthier one had boiled milk. The sicklier one did better than the healthier one.

These data certainly seem to show that there are cases of sick children where raw milk gives better results than boiled milk; at the same time the evidence already given shows that many children who are suffering from mal-nutrition do extremely well upon boiled milk. It would not appear to be justifiable to draw conclusions in regard to the relative nutritive properties of raw and boiled milk in the treatment of healthy babies, as a result of these experiments of Hohlfield's, where only four babies were given boiled milk, and of those, two had an apparent idiosyncrasy against boiled milk and vomited it after administration.

Vincent (65) (1906) has published 20 cases of markedly severe malnutrition in infants, which were fed upon raw milk and did very well. These cases were fed upon the percentage method of feeding, under constant supervision, and often of change of the percentage of ingredients. No case was fed upon boiled milk of a similar percentage mixture.

The work of most value is that of Finkelstein (28) (1907), who carried out prolonged experiments upon both healthy and atrophic children, as well as upon those suffering from acute disturbances. As far as possible the same number of children were fed upon raw and boiled milk in each of these groups. The milk used was from the best dairy in Berlin, and was of excellent quality; the same milk was used for administering raw and for boiling.

The results are best seen by reproducing Finkelstein's table of results.

GROUP I. (A) Healthy children, that is children with no apparent signs of disease; under eight weeks of age, and having an average weight of 3,600 grammes.

—	Raw milk.	Boiled milk.
Number of children	29	19
Average number of days of observation	34	27
Good result—good progress, no disturbance	10 = 34·4 per cent.	10 = 52·6 per cent.
Daily increase	15·5 grammes.	19·8 grammes.
Fair result—progress not so good, no disturbance ...	7 = 24·1 per cent.	2 = 10·5 per cent.
Daily increase	7·6 grammes.	8·0 grammes.
Bad result—loss of weight, or appearance of acute disturbance.	2 = 41·5 per cent	7 = 37 per cent.

(B) Twelve children were fed periodically with raw and boiled milk. The results were—

—	Average length of feeding,		Daily increase.	
	Raw milk.	Boiled milk.	Raw milk.	Boiled milk.
	days.	days.	grammes.	grammes.
Raw and boiled milk equally good 3 = 25 per cent.	33	17	16·3	14·7
Raw and boiled milk equally bad 4 = 33 per cent.	32	22	— 5·1	—11·0
Raw milk better than boiled 3 = 25 per cent.	27	20	13·5	—10·0
Boiled milk better than raw 2 = 16 per cent.	25	19	3·5	15·5

GROUP II.—Atrophic children, older than Group I., markedly below weight, but without other symptoms of disease. Raw and boiled milk given periodically.

—	Average length of feeding.		Daily increase.	
	Raw milk.	Boiled milk.	Raw milk.	Boiled milk.
	days.	days.	grammes.	grammes.
Raw and boiled milk equally good, two cases.	37	36	20	16
Raw and boiled milk equally bad, one case.	28	33	5	1

GROUP III.—(A) Children with digestive disturbances, showing strong local and, usually, general symptoms. The quantity, &c., of the food was the same in both cases.

—	Raw milk.	Boiled milk.
Number of children	25	16
Average length of observation in days ...	38	26
Improved and did equally well	15=60 per cent	16=68·8 per cent.
Did not improve	10=40 per cent.	5=31·2 per cent.

(B) Nine children were fed periodically with raw and boiled milk.

—	Average length of feeding.		Daily increase.	
	Raw milk.	Boiled milk.	Raw milk.	Boiled milk.
	days.	days.	grammes.	grammes.
Improved equally well with raw and boiled milk, 4=44 per cent.	33	22	19	19·3

No improvement with either raw or boiled milk, 3=33 per cent.
Improved on raw milk, after failure on boiled milk, 1=11 per cent.
Improved on boiled milk, after failure on raw milk, 1=11 per cent.

Finkelstein concludes that "no definite distinction between the "results obtained by feeding upon raw and boiled milk respectively, "could be detected." If these figures of Finkelstein's are examined a little further, it is seen that in Group I. (A) there is a balance in favour of the boiled milk, whereas in the other groups there is a balance which is slightly in favour of the raw milk.

Finkelstein also quotes three cases of anæmia with splenomegaly, who showed no improvement with very prolonged feeding upon raw milk, but which subsequently recovered on boiled milk.

Plantenga (^{51}) (1910) treated children with digestive troubles with both raw and boiled milk, and from a study of the weight-curves was unable to find any evidence of the superiority of the one over the other, or *vice versâ.*

Reviewing the clinical evidence as a whole, it seems impossible to draw any other conclusion than the following :—

If healthy or not markedly atrophic children are considered, no great difference can be detected between the nutritive values of raw and boiled milk, given always that the quality of the milk is the same.

If markedly atrophic or sick children are considered, there appears to be a good deal of idiosyncrasy, some doing well only upon the one form of food, and some only upon the other. The value of much of the clinical work on this subject is very much reduced by the absence of control cases. In this respect the earlier work is especially deficient ; as also in regard to the details of the milk given.

Metabolism Experiments.—A considerable number of metabolism experiments have been carried out upon infants with a view to ascertaining the difference in the values of raw and boiled milk respectively. These, however, are somewhat beyond the scope of this report, since if pushed to their conclusion these experiments really lead towards the discovery of those constituents of the milk, if any, which are injured by boiling. It is impossible in any one experiment to estimate the metabolism of all the constituents of the milk, and one or two must therefore be selected. Hence by these experiments information is gained as to the nutritive value of different constituents of raw and boiled milk, and not of raw and boiled milk as a whole, which last alone is being dealt with in this report.

It is, however, very usual to regard nitrogenous metabolism as giving considerable indication of the general state of health of the body, and it may therefore be of advantage to deal briefly with a few of the experiments which have been carried out in this direction.

Bendix (4) (1894) fed children of from 1½–2 years old upon boiled and sterilised milk during alternate weeks. The children also received chocolate, apple jelly and bread. The only difference which could be detected in the metabolism of the infants was a decrease in the nitrogen-absorption of ·4 per cent. with sterilised milk, and an increase in the fat absorption.

It may be noted that the children were no longer infants.

Lange (43) (1895) worked upon dyspeptic infants, and found that the dyspeptic infant passes a larger amount of fæces, and that the total content of nitrogen although absolutely greater, gives a lower percentage, than in healthy babies.

He used nine artificially-fed babies, and one breast-fed baby, the former being fed upon modified cows' milk, sterilised. He found that the assimilation of nitrogen with the cow's milk was almost as good as with mothers' milk, being 95·4 per cent. as against 97·57 per cent. of the nitrogen taken in. The experiments were conducted over one or two days only, and it is regrettable that only one breast-fed baby was taken.

Koplik (40) (1895) fed four children upon different varieties of milk:—

- 1st child, 3 months old, was fed upon the breast and sterilised milk for 6 days.
- 2nd child, 3 months old, received sterilised milk for 6 days.
- 3rd child, 3 months old, received—
  - (1) Pasteurised milk (70°C.) for 7 days.
  - (2) Sterilised milk (100°C.) for 7 days.
  - (3) Raw milk for 7 days.
- 4th child, 5 months old, received—
  - (1) Boiled milk for 3 days.
  - (2) Sterilised milk for 7 days.

He found great variations in the daily amounts of nitrogen in the fæces from day to day, in all the methods of feeding, and concluded that in this respect no difference could be detected between the different methods of feeding with cows' milk.

Here again therefore it seems impossible to detect any difference between the nutritive values of raw and boiled cows' milk in the feeding of infants.

The alleged production of Rickets and Infantile Scurvy by boiled milk.—It will be of advantage to examine briefly the main objections which have been raised against the use of boiled milk as opposed to raw.

These consist in the alleged production of rickets and infantile scurvy, as a result of the prolonged use of boiled or sterilised milk.

It would be impossible in this report to discuss this subject at length, but some points can be dealt with.

Many thousands of children are fed every year both in this country and on the Continent, on pasteurised, boiled, and sterilised milk. No records are available of a very great number of these cases, but a vast mass of material has gradually been accumulating at the large Infant Consultations which have now been in existence for a considerable number of years in many of the great cities of Europe.

So far as I can ascertain there is no consultation of any size in Europe where the infants attending this institution are consistently fed upon raw milk. They are fed upon pasteurised, boiled or sterilised milk. It may be again pointed out, that owing to inaccuracy of nomenclature milk is often described as "pasteurised" which is really boiled, or just brought up to boiling point of water, and milk which is called "sterilised" is really only "boiled."

If, however, boiled or sterilised milk really produce either rickets or Barlow's disease (infantile scurvy) then these troubles certainly ought to be prevalent among the children attending the consultations where these forms of diet are used. A large number of reports of these consultations can now be obtained giving accounts of the health of the children, who are seen at frequent intervals. A few of these reports only need be considered. Thus in 1904 Mons. Variot (64) issued the report of his consultation at Belleville, near Paris, where the children were fed on sterilised milk (heated to 108°C.).

In 3,000 cases included in this report no case of Barlow's disease is recorded.

A few cases which had been either over-fed or had received farinaceous food while still too young developed rickets, but not Barlow's disease, and all the other children did very well.

A similar testimony is borne by Mons. Bresset (15), the presiding physician at one of the consultations in Paris. Among over 2,000 cases fed on sterilised milk, no case suspicious of Barlow's disease was seen. Rickets was rare, and seems altogether to be far from common among the Parisian children.

Budin ("The Nursling," Chap. 9) says: "As for the so-called "'infantile scurvy,' which is alleged to follow the use of sterilised "milk, I have heard a very great deal about it during the last few "years, but I am still looking for my first case."

In Berlin thousands of children attend the municipal consultations and Barlow's disease is rare. (Private information from Dr. Ballin.) Rickets is a very common disease in Berlin, but it will be seen in Part IV. (p. 35) that this can hardly be attributed to the feeding upon boiled milk.

As regards the question of the occurrence of Barlow's disease and rickets, Escherich (25) says: "Infantile scurvy (Barlow's disease) "I have not once seen among all the many thousands of children "fed artificially and with sterilised milk, which passed through my "hands in München and Graz." And later "As regards the much "disputed question of rickets, in my experience it is hardly less "frequent in breast-fed babies than in bottle-fed ones: the worst "degrees of rickets are, however, only met with in the latter class."

It would appear therefore not to be correct to assert that rickets and Barlow's disease are caused by the administration of either boiled or sterilised milk, as such, although when Barlow's disease occurs the infants have often been fed upon sterilised milk. They have also often had an undue amount of farinaceous food.

The cases published by Cheadle (19) in 1878 had all been fed upon farinaceous food, and if the literature be studied it will be found that these are by no means isolated instances.

La Fetra (27) and others quote cases of Barlow's disease occurring in breast-fed babies, and in children who had been fed upon various kinds of food. In order to account for its appearance in the breast-fed babies, the disease was attributed to the state of health of the mother.

Medical literature contains no lack of clinical reports upon cases of this disorder, and the almost dramatic manner in which a cure can be effected probably tends to give infantile scurvy a more prominent place than is usually accorded to a disease which is far from common, and for which definite and satisfactory lines of treatment are known.

Although Barlow's disease is not confined to children fed upon one class of food, at the same time the great majority of patients with this disease do appear to have been fed upon milk which had been subjected to prolonged boiling or sterilising, or to milk which had been manipulated to an unusual extent.

In the recent literature Lust (44) quotes six cases seen by him within a short time in private practice, which were suffering from infantile scurvy. All these cases had been fed either upon sterilised milk, or upon Backhaus milk (a somewhat elaborately prepared milk) and he considered that there was evidence that it was associated with over-feeding.

Many other similar cases might be quoted from the literature of the subject.

The Medical Society of Geneva recently held an inquiry into the frequency of the occurrence of Barlow's disease in Switzerland (7). Papers of inquiry were sent to the medical men of the district, and 71 answers were received.

Of the 71 only five had seen cases of the disease, and only 10 cases were reported, of which six were reported from Geneva itself.

Of these 10 cases nine had been fed either upon special forms of milk or upon milk sterilised at high temperatures. The tenth child had been fed upon milk prepared in a Soxhlet apparatus.

It seems possible that the length of time which the milk is kept before consumption may be of importance. Thus, Carel (28) quotes the case of a child which was taken away from home with a large supply of sterilised milk, sufficient for several weeks. Apparently the child showed symptoms of Barlow's disease, which disappeared with the arrival of a fresh supply of sterilised milk. As this fresh supply became stale the symptoms reappeared and the child was brought for medical advice, and the case diagnosed as one of infantile scurvy. The relations stated that the symptoms were identical with those which had disappeared when the fresh supply of sterilised milk was given to the child.

Plantenga (54) has published an account of an outbreak of Barlow's disease which occurred among the children of his consultation. During one year the milk which was given out at his consultation was pasteurised over night by heating at 70° C. for ½ hour. The milk, therefore, although not boiled was yet subjected to prolonged heating. In the morning this milk was further heated, being boiled for five minutes in a Soxhlet's apparatus. In that year 23 cases of Barlow's disease developed among the babies of the consultation, out of a total number of 200.

As a result of this outbreak the routine of the milk preparation was changed, and the morning's milk was merely pasteurised without being kept. No case of Barlow's disease occurred on this form of milk. Plantenga attributed the occurrence of the trouble to the length of time the milk had been kept before it was used, and he pointed out that the value of raw milk in certain cases may arise from the fact that it is given sooner after milking.

The available clinical evidence in connection with the milk of a foreign species, cannot be said to show any marked difference in the nutritive properties of raw and boiled milk when used in the treatment of infants in health, or suffering from nutritive disturbances.

As regards "sterilised" milk or milk which has been heated over prolonged periods or at high temperatures, it appears that it also can be used with good results. At the same time it seems probable that sterilised milk which has been kept or milk which has been subjected to somewhat extensive manipulations may be one of the factors in the production of infantile scurvy. The number of cases of this disease which arise in children fed upon sterilised milk, and other forms of milk is, however, so small in comparison to the number of infants thus fed, that it does not seem reasonable to suppose it to be the only factor concerned.

Summary of the Evidence of Part III.—The clinical evidence as to the nutritive value of boiled human milk as an infants' food is on a small scale; but the infants who have been fed on such milk over prolonged periods (cp. Thiemich) show equally good nutrition with the breast-fed infants. A few observers working with scanty material and over a short period have arrived at a different conclusion.

There is considerable divergence in the conclusions based on experience of different observers, as to the relative nutritive value of raw and boiled cows' milk as a food for infants. Some observers claim to have obtained better results by feeding infants on raw than on boiled milk. Some have found no detectable difference between the two groups; and others even claim an advantage in favour of boiled milk. The balance of evidence may be said not to show any decided superiority on the side of either raw or boiled cows' milk as a food for infants.

PART IV.—METHOD AND RESULTS OF WORKING UP THE BERLIN MATERIAL.

The material for this research was obtained, as already mentioned (p. 4), from the Infant Consultation of the Naunyn Strasse in Berlin. This consultation is conducted by Dr. Ballin, to whom I am deeply indebted for permission to use his material.

Source of the Material.—Six years ago, infant consultations were started by the municipality of Berlin, under the auspices of a special fund, the Schmidt-Gallisch Stiftung.

Four were first started, and then another, and finally two more, thus making seven in all, in different parts of Berlin.

Each of these consultations is in charge of a medical officer who has made a special study of the diseases and ailments of children. The attendance is so large that assistants have been appointed to assist the medical officer in the discharge of his duties. The consultations are held daily, and at the Naunyn Strasse (where the material here dealt with was obtained) the average daily attendance is about 100 babies.

Each Consultation has its own staff of health visitors attached. These are women who have been thoroughly trained for work among children, and are appointed by the municipality to visit the homes of the babies who are brought up to the consultation. Their duty is to instruct the mothers in the general care and hygiene of the infant in accordance with the directions given by the medical officers at the consultation.

The clientèle of the consultation consists exclusively of the working classes. The fathers of the children who are brought up to the consultation are for the most part earning about 20–30 marks a week (see p. 41 and table VI.).

Parents whose income is over 40 marks a week are expected to pay a private doctor, and are only allowed to attend in very special cases.

The attendance at the consultation is entirely voluntary, except in a few cases where the babies are illegitimate, and are boarded out under the Poor Law authorities; here the consultation is used as a means of keeping the baby under medical supervision.

The simpler ailments of children are dealt with at the consultation, but sick children in need of in-patient treatment are referred to the hospital.

Breast-feeding is actively encouraged, and the great majority of the children are breast-fed. Small nursing-bonuses are given to the nursing mothers. (Usually about two marks a week.)

The Milk Supply to the Consultation.—Regulations to ensure the quality and purity of the milk for the babies attending the consultation were drawn up when the consultations were first started, and tenders were invited from dairies who were willing to comply with the regulations. The milk was required to be of a high standard, and was subjected to regular chemical and bacteriological examination by the municipality.

Two years ago, the town of Berlin started its own dairies outside Berlin, and all the municipal institutions are now supplied from these municipal dairies.

In these dairies 200 cows are kept which are tubercle-free, and the farm is conducted upon all the model lines of an up-to-date dairy.

The milk is examined chemically and bacteriologically, and I was informed that the fat content is never less than 3 per cent., and the bacterial count varies from 20,000 to 30,000 per c.c. The milk is thus one of great purity.

After milking, the milk is rapidly filtered, bottled, and cooled to 3–4° C., at which temperature it is kept in the cold chamber. It is sent off as soon as possible by special train to Berlin, where it is delivered in cooled vans to numerous centres (about 80) in various parts of the town, whence it is fetched by the mothers of the infants attending the consultation, who are being artificially fed.

Milk is not given out by the consultation, except in a very few cases in which for some reason a plain milk dilution is not considered suitable.

The milk is paid for in full if possible by the parents of the babies, or in part if the social conditions are poor. Each case is dealt with on its own merits on the report of the health visitor, and in some cases the milk is given free. No child is allowed to have a deficient supply of milk as a result of the poverty of its parents.

Over-feeding is avoided by the amount ordered by the medical officer being written on a separate card, which the mother has to show to the dairy when the milk is fetched. The card is valid only for a certain number of days (usually 7–10 days, according to the discretion of the medical officer of the consultation), being stamped with the date up to which the milk may be given out by the dairy. After that date the mother must bring the baby up to the consultation in order to have the card stamped for a further period. The food of the infant and the milk of the municipality are thus kept under control.

The milk is ordered to be diluted on much the same lines as are followed in this country. The preparation of the milk in the home is under the care of the health visitor who personally instructs the mother in this important matter. The milk is fetched from the dairy, is cooled, and is directed to be brought to the boil, and to be allowed to froth up twice. It is then at once covered, and placed in a vessel of cold water. In hot weather the water is directed to be changed frequently. Thus every care is taken of the food for the artificially-fed baby.

The Notes taken at the Consultation.—Full and careful notes are taken at the Consultation, and entered on special sheets. The notes show the date of the child's birth, the date of its first attendance at the consultation, the feeding of the infant before its first attendance, the health of the parents, the number of children in the family, the wages and the general social condition of the family, the legitimacy or illegitimacy of the infant, and any other point which may require noting.

During the attendance of the infant the dates of its attendance, the weight at each attendance, the directions as to feeding, and the medical notes as to the child's health are all entered upon these sheets. In addition the remarks of the health visitors as to the general condition of the home, the baby, and the milk are entered after each visit. The visits are made at frequent intervals.

The records are thus very complete, and many thousands of such charts are stored at the consultation in the Naunyn Strasse.

In copying the data required from these consultation sheets, the following points were taken—

(1) The date of the child's birth.
(2) The date of its first attendance at the consultation.
(3) The legitimacy or otherwise of its birth. (Nearly all were legitimate.)
(4) The wages of the father.
(5) The number of children in the family.
(6) The weight at each attendance.
(7) The date of each attendance.
(8) The medical notes.

The last three points were tabulated in columns in the following manner, the day of age of the children being worked out afterwards and carefully checked—

Date.	*Day of Age.*	*Weight.*	*Remarks.*
10th August, 1908.	20	3,300 grammes.	Breast, six times a day.
20th August, 1908.	30	3,500 „	Bronchitis.

and so on.

Selection of Material.—It will be remembered that the object of the investigation was to compare the nutrition, as measured by weight, of infants fed on the breast and on boiled cows' milk. The selection of the material presented several points of difficulty. It was necessary to deal with two main series of infants :—

(1) Healthy babies of the average artisan class, fed upon milk in various forms, in order to have a control consisting of the average baby.

(2) Healthy babies of the same class but fed only upon boiled cows' milk, in order to study the difference, if any, produced upon the average baby of the class by feeding it exclusively upon boiled milk, as compared with the infant of class (1).

In considering the babies of this first class, it appeared that far the larger portion were fed upon the breast, and that all the others were fed upon boiled cows' milk. There were a very few cases of mixed feeding upon the breast and on boiled milk, lasting over a few weeks.

This was of course foreseen, since the material which was to be used was that of an infant consultation where the babies were if possible fed upon the breast, and failing that upon boiled milk.

Hence if the babies attending the consultation had been taken seriatim, regardless of the variety of feeding they were receiving, and had been utilised for preparing control statistics under the first head given above, the results would have been complicated by the presence of a considerable number of babies of the second class, which was required to be a class by itself. It was decided to exclude from the control series all babies who had received less than four months breast feeding, taking into consideration further points described below.

Further, in order to eliminate the effects of temperature it was considered advisable as far as possible to take the babies of both classes from the same years. They were all taken from the years 1907–08, and 1908–09, and about half the cases taken came from each of these years.

The most serious difficulty in the selection of the material arose from the varied age at which the children commenced attendance, the varied length of attendance, and the question of the serious illness or death of the child during its attendance at the consultation or soon after the cessation of its visits. It was finally decided to exclude (*a*) all infants who were over four months of age at the time of their first attendance ; (*b*) all who did not attend over a period of at least four months with regularity ; (*c*) all babies who were suffering from constitutional diseases, or who developed such during their attendance at the consultation ; and (*d*) to exclude all babies who died during their attendance at the consultation, owing to the difficulty of ascertaining how far they had been initially healthy babies.

Figures kindly supplied to me by Dr. Ballin, which are not here reproduced, confirm the conclusion, at which I had independently arrived, that the exclusion of babies mentioned under (*d*) above did not materially affect the statistics.

Analysis of the Material.—The material has been analysed with the view of showing the age of the babies at their first attendance at the consultation, and also how long they remained under the care of the medical officers of the consultation.

The day of age of the babies has been worked out for each attendance (as already explained) upon the copies of the consultation charts.

A table was then constructed of the day of age of the children at their first attendance and of the age at which they ceased attending the consultation.

It was then apparent that the duration of attendance of the infants which were brought up at the same day of life, was so varied that it would be impossible to construct a table of any reasonable form which would give the length of attendance in weeks, much less days. Further the calculation of the age of an infant in weeks becomes somewhat inaccurate towards the later months of the first year, owing to the difference between the calendar and lunar month. Thus 36 weeks is nearer eight calendar months than nine, and thus "weeks" gives a false impression of the age. A month of 30 days was therefore taken as the unit of attendance in the table given below. Thus the age of life from 180-210 days is taken as from the 6–7 month of age. Any child leaving the consultation between those days of life, comes under the column dealing with the 6–7 month, and so on. Many of the cases attended up to 13 months of age or even rather over but they have not been dealt with over 12 months of age.

The unit of grouping for the first attendances has been taken as one week (seven days), since where only 120 days (four months) (the limit of age allowed in this research for the first attendance) were being dealt with, (see p. 31), 30 days is evidently too large a unit. As 120 days is between 17 and 18 weeks, 18 weeks have been entered upon the table, although 120 days was the figure taken in collecting the material.

It seemed of interest to give the actual number of children who attended for the first time upon each day of life, and therefore a special column (column II.) gives the actual numbers of children who were brought up for the first time on each consecutive day of age of the period of seven days under consideration.

The length of time of breast-feeding in both series evidently requires consideration, but it could not be given in the same table, and is therefore given separately below. (See pp. 34, 35.)

In the tables given (Tables I. and II.)—

Column I. gives the age of the babies at the first attendance at the consultation grouped in periods of one week (seven days).

Column II. gives the actual number of children who were brought up for the first time on each of the consecutive days, of the period of seven days of column I. Thus the number of children who were 25 days old when first brought up would be given by the fourth figure of the fourth period of seven days.

Column III. gives the ages of the children on leaving the consultation which had been first brought up at the age given in column I. grouped in periods of 30 days.

Column IV. gives the total number of children which were brought up for the first time during the period of age given in column I.

TABLE I.

Showing the age of first attendance and of leaving the Consultation of the Babies of the Control or Breast-fed Series.

I. Age in weeks.	II. No. brought at each day.	III. Age on leaving (in months).							IV. Total in each week.
		4-5.	5-6.	6-7.	7-8.	8-9.	9-10.	10-12.	
1	3. 2. 0. 3. 0. 4. 4.	2	1	1	0	4	0	8	16
2	5. 7. 8. 6. 6. 7. 10.	9	4	5	3	1	3	24	49
3	8, 11, 9, 15, 12, 5, 7.	8	11	5	6	8	9	20	67
4	9. 4 3. 8. 0 3. 4.	2	3	7	5	1	2	11	31
5	1. 2. 4. 5 2. 3. 2.	—	1	3	1	2	1	11	19
6	5. 2 3. 5. 2. 2. 3.	—	6	3	1	3	0	9	22
7	3. 1. 2. 3. 4. 2. 2.	—	4	1	5	1	2	4	17
8	1. 2. 1 0. 3. 3. 0.	—	3	2	2	1	0	2	10
9	2. 2. 3. 4. 3. 2. 2	—	3	6	2	2	1	4	18
10	1. 1. 1. 1. 2. 0. 1.	—	—	3	1	1	0	2	7
11	2. 4. 3. 3 0 0. 1.	—	—	4	1	1	1	6	13
12	3. 0. 0. 0. 3. 5. 1.	—	—	—	4	1	2	5	12
13	2. 1. 0. 1. 2. 0. 2.	—	—	—	1	0	2	5	8
14	1. 1. 0. 0. 0. 0. 2.	—	—	—	1	0	1	2	4
15–18	——	—	—	—	—	3	1	3	7
		21	36	40	33	29	25	116	300

This table shows that comparatively few of the children attended only the minimum period of 120 days. The actual number of such cases was 28 in all, made up of

11 babies whose first attendance was at an age of from 1–30 days.
9 ,, ,, ,, ,, ,, 30–60 ,,
7 ,, ,, ,, ,, ,, 60–90 ,,
1 baby ,, ,, ,, ,, 90–120 ,,

Twenty-one of these babies appear in the first column of column III which gives the numbers who attended up to the age of from 120–150 days, and seven babies in the other columns of column III.

Table II.

Showing the age of first attendance and of leaving the Consultation of the Babies of the boiled cows' Milk Series.

I. Age in weeks.	II. No. brought at each day.	III. Age on leaving (in months).							IV. Total in each week.
		4–5.	5–6.	6–7.	7–8.	8–9.	9–10.	10–12.	
1	2. 0. 0. 1. 2. 0. 2.	1	0	1	1	0	0	4	7
2	2. 1. 1. 1. 5. 7. 2.	0	1	4	2	0	3	9	19
3	6. 4. 3. 8. 2. 4. 2.	0	1	3	0	1	2	22	29
4	6. 2. 4. 4. 4. 4. 3.	3	3	2	1	3	1	14	27
5	1. 1. 3. 1. 4. 2. 4.	0	0	2	0	1	3	10	16
6	1. 2. 1. 2. 4. 1. 1.	3	0	2	3	0	1	3	12
7	1. 2. 2. 2. 2. 0. 5.	—	—	1	2	0	1	10	14
8	1. 3. 2. 3. 3. 2. 4.	—	—	—	2	0	2	14	18
9	2. 0. 3. 4. 2. 0. 2.	—	—	2	1	2	2	6	13
10	0 3. 2. 1. 1. 0. 2.	—	—	—	1	0	1	7	9
11	0. 3. 1. 1. 1. 0. 2.	—	—	1	2	2	1	2	8
12	0. 3. 1. 0. 0. 1. 1.	—	—	—	2	0	0	4	6
13	1. 2. 4. 0. 1. 1. 1.	—	—	1	1	2	1	5	10
14	1. 1. 1. 1. 1. 1. 0.	—	—	—	—	—	1	5	6
15–18	——	—	—	—	—	3	1	6	10
		7	6	19	18	13	20	121	204

Here still fewer children attended only the minimum number of days, but three attended a few days under the 120.

Including these three, in all nine babies attended the minimum time and these were as follows :—

4 babies whose first attendance was at an age of from 1–30 days.
3 " " " " 30–60 "
2 " " " " 60–90 "

Seven of these children appear in the first column of column III. where the numbers who attended up to the age of from 120–150 days are given.

These tables show that the great majority of children attended well over the minimum period of 120 days, or four months. All the children who attended up to from 8–9 months of age, must evidently have attended over four months.

On this basis adding together the figures in the last three columns of column III. it is seen that in the control or breast-fed series 170 children attended over four months. While in the other series or boiled milk series 154 babies attended over four months. This, however, does not give the full number, since in the other columns of column III. there are many children who had already attended many more than fonr months at the age of eight months.

Length of Breast-feeding.—In the control or breast-fed series of babies, all had been breast-fed for at least four months; about 10 of these children were having *allaitement mixte* part of this time, but as it is impossible to take every minor point into consideration this was ignored. Moreover this series was taken as a general control, and not as a purely breast-fed series.

Age of Weaning in Control Series.—In this series there were in all 52 babies who were weaned between the ages of four and six months. The rest were weaned at about nine months of age.

Age of Weaning in the Series of Babies fed upon Boiled Cows' Milk.—In this series, out of 204 babies—

78 had never been breast-fed.
41 had been breast-fed for periods varying from 1–8 days.
15 had been breast-fed for periods varying from 9–14 days.
40 had been breast-fed for periods varying from two weeks to two months.
17 had been breast-fed for ~~periods of~~ over two months, but under four months.
13 had been breast-fed once or twice a day for a few weeks after birth, but otherwise had had only boiled cow's milk.

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Analysis of the Medical Notes upon the Health of the Babies which attended the Consultation.—Before proceeding to the results obtained from the material itself the health of the babies under review must be considered. It has already been stated that the minor ailments although noted upon the charts, have been ignored. But the notes upon the condition of the babies as regards rickets are of interest. These are of course only of use where the child attended up to from 9–10 months of age. There are a few notes as to rickets in the children under this age, but these will be neglected, and only the notes of the older children will be considered. Every baby who is still attending the consultation at the age of 12 months, is examined for rickets, and if no note is entered it means that no evidence of this trouble could be detected. (Dr. Ballin explained to me that this was the usual routine of the consultation.)

The remarks fall into several main headings, including teeth, no teeth, rickets, rickets with teeth, and rickets but stands.

Taking the babies who were still attending at the age of from 10–12 months, it is seen from the analysis of the cases just given that 116 babies of the control or breast-fed series, and 121 babies of the boiled milk series, were still attending at that age.

Only the remarks as to the presence of rickets need be considered, the other cases being taken as healthy. Tabulating the notes the following figures are obtained—

Number of children noted as showing

	In the control series.	In the boiled milk series.
No teeth ...	2	5
Rickets ...	7	15 and 1 ?
Rickets with teeth	16 and 2 ?	11 and 2 ?
Rickets but stands	5	5 ? rickets.
	30 and 2 ?	31 and 8 ?

If failure to cut a tooth by the age of 10 months be taken as a sign of rickets, then out of 116 babies of the first series there were 30 babies who showed definite evidence of rickets, and 2 where it was doubtful.

In the boiled milk series there were 31 out of 121 babies which had definite rickets, and 8 which were doubtful.

Whichever way the figures are taken they are too close to admit of any deductions as to any greater incidence of rickets as a result of feeding upon boiled milk as compared with breast-feeding. Nor are a series of 116 and 121 cases large enough for this purpose. All that can be said is that, as far as the figures of these particular cases go, there is no evidence of any greater frequency of rickets among the babies fed upon boiled cows' milk than in those fed upon the breast. The percentage of Berlin babies which are suffering from rickets is known to be high.

In this connection compare Escherich (p. 26).

Results obtained by working up the Material.—In working up the material which had been obtained from the Berlin consultation the first point was to add the weights of all the babies of each series at the same day of life, and then divide by the number of babies weighed, thus obtaining the average weight of the babies under consideration at the same day of life.

The average weights so obtained showed considerable inequalities, and the number of average weights thus obtained was unwieldy and cumbersome. It was therefore decided to take the unit of age as eight days, and to group together all the weights of the babies from 1–8, 9–16 days of age and so on up to 368 days of age.

The total weights of all the babies which were weighed in each eight-day period of life were divided by the total number of babies weighed. Thus the average weight of all the babies of each series for consecutive periods of eight days of life up to one year of age was obtained, the inequalities of the averages for each day being thus smoothed out and the number of average weights reduced to a convenient number for plotting on a curve.

No serious overlapping of weighings was produced by this method, since the babies were usually brought up at intervals of eight or ten days, so that it was only in exceptional cases that the weight of the same baby was recorded twice in any period of eight days.

Table III. shows the results obtained by thus working up the babies of the control or breast-fed series, and Table IV. the corresponding results for the babies of the boiled cows' milk series.

In these tables

Column I. shows the age in periods of eight days of the babies considered.

Column II. given the number of observations, *i.e.*, the number of babies which were weighed upon each of the consecutive days of each period of eight days; the figures being given in chronological order.

Column III. gives the total number of observations (weighings) in each period of eight days, as given in column I.

Column IV. gives the average weight of the babies of each series for each period of eight days corresponding to the period of column I.

NOTE.—As regards Column II., in collecting the weights of the babies the weights were worked up for each day separately up to 100 days of age, from 100–224 days of age they were worked up in two-day periods, and from 224–368 days the weights were worked up in four-day periods. This is shown in the tables.

The average weights found, and shown in Tables III. and IV. are plotted on Diagram I.

The total number of observations worked up for the cases in Series I. amount to 6,297 and for Series II. to 5,444.

TABLE III.

Showing the average weights of the babies of the control or breast-fed series, grouped in periods of eight days, and the number of observations made.

I. Age in days.	II. No. of observations on each day.	III. Total No.	IV. Average Weight.
			Grammes.
1–8	3. 2. 0. 4. 0. 4. 6. 5.	24	3,185
9–16	10 10 9. 11. 11. 12 12. 22.	97	3,317
17–24	16. 21. 23. 19. 19. 20. 13. 13.	144	3,507
25–32	20. 24. 16 19. 20. 19. 31. 18.	167	3,746
33–40	16. 19. 19. 27. 23. 25. 18. 15.	162	3,939
41–48	29. 18. 27. 19. 19. 24. 29. 27.	192	4,119
49–56	18. 19. 33. 25. 19. 18. 26. 17.	175	4,291
57–64	29. 29. 31. 24. 30. 29. 25. 27.	224	4,443
65–72	23. 20. 35. 27. 27. 25. 27. 35.	219	4,638
73–80	26. 24. 28. 19. 32. 34. 31. 21.	215	4,737
81–88	34. 40. 27. 29. 31. 25. 26. 26.	238	4,937
89–96	35. 22. 41. 32. 27. 30. 23. 22.	232	5,079
97–104	26. 27. 25. 33. 61. 52.	224	5,191
105–112	54. 58. 45. 60.	217	5,380
113–120	54. 54. 58. 50.	216	5,666
121–128	60. 57. 61. 53.	231	5,659
129–136	52. 61. 59. 55.	227	5,757
137–144	60. 48. 56. 56.	220	5,929
145–152	47. 50. 51. 43.	191	6,033
153–160	47. 56. 45. 43.	191	6,237
161–168	50. 50. 45. 39.	184	6,274
169–176	47. 47. 48. 40.	182	6,312
177–184	41. 41. 46. 43.	171	6,434
185–192	41. 32. 27. 47.	147	6,458
193–200	32. 28. 41. 27.	128	6,664
201–208	32 37. 29. 35.	133	6,709
209–216	31. 31. 30. 28.	120	6,734
217–224	32. 37. 35. 32.	136	6,798
225–232	54. 56.	110	6,778
233–240	55. 54.	109	6,886
241–248	58. 42.	100	6,891
249–256	42. 47.	89	7,118
257–264	38. 40.	78	7,276
265–272	46. 40.	86	7,217
273–280	43. 38.	81	7,388
281–288	36. 41.	77	7,281
289–296	28. 35.	63	7,608
297–304	34. 38.	72	7,567
305–312	36. 32.	68	7,801
313–320	22. 36.	58	7,555
321–328	32. 26.	58	7,753
329–336	37. 20.	57	7,704
337–344	26. 22.	48	7,752
345–352	21. 22.	43	8,034
353–360	22. 15	37	8,077
361–368	19. 15.	34	8,274

TABLE IV

Showing the average weights of the babies of the boiled cows' milk series, grouped in periods of eight days, and the number of observations made.

I. Age in days.	II. No. of observations on each day.	III. Total No.	IV. Average weights.
			Grammes.
1–8	2. 0. 0. 1. 2. 0. 3. 2.	10	3515
9–16	2. 3. 2. 6. 7. 5. 9. 6.	40	3090
17–24	7. 11. 6. 15. 11. 13. 4. 9.	76	3358
25–32	15. 17. 15. 14. 14. 13. 11. 14.	113	3472
33–40	15. 14. 15. 14. 17. 17. 15 18.	125	3708
41–48	12. 13. 21. 13. 17. 15. 23. 17.	131	3848
49–56	15. 23. 16. 16. 17. 21. 17. 18.	143	3991
57–64	23. 14. 26. 21. 18. 12. 19. 19.	152	4082
65–72	21. 20. 21. 20. 15. 23. 15. 20.	155	4240
73–80	22. 20. 24 20. 20. 22. 22. 9.	159	4407
81–88	25. 16. 26. 31. 20 24. 26. 28.	196	4486
89–96	18. 13. 34. 16. 26. 21. 32. 25.	185	4628
97–104	15. 22. 30. 10. 55. 30.	162	4814
105–112	47. 49. 39. 38.	173	4935
113–120	46. 46. 44. 42.	178	5052
121–128	41. 53. 40. 45.	179	5171
129–136	46. 38. 46. 41.	171	5326
137–144	41. 45. 43. 35.	164	5436
145–152	40. 40. 41. 41.	162	5569
153–160	47. 40. 37. 36.	160	5669
161–168	36. 45. 40. 27.	148	5831
169–176	45. 41. 39. 39.	164	5915
177–184	29. 38. 41. 36.	144	6146
185–192	33. 33. 37. 37.	140	6242
193–200	36. 37. 34. 31.	138	6319
201–208	67. 62.	129	6475
209–216	77. 53.	130	6467
217–224	68. 65.	133	6677
225–232	66. 67.	133	6721
233–240	52. 59.	111	6770
241–248	62. 52.	114	7010
249–256	53. 70,	123	7112
257–264	37. 57.	94	7204
265–272	53. 41.	94	7274
273–280	39. 41.	95	7347
281–288	48. 43.	91	7481
289–296	37. 48.	85	7512
297–304	40. 39.	79	7610
305–312	42. 42.	84	7788
313–320	28. 34.	62	7765
321–328	40. 27.	67	7887
329–336	36 28.	64	8281
337–344	28. 31.	59	7985
345–352	20. 23.	43	8194
353–360	27. 21.	48	8281
361–368	25. 13.	38	8613

Analysis of the curves of Diagram I.—Diagram I. shows at once that a considerable divergence between the two curves starts in the early days of life, and continues well-marked up to about the 208th day, after which it disappears fairly rapidly. The question suggested by these curves is,—Is the difference between the average weight of breast-fed and of babies of the same age fed upon boiled cows' milk due to the method of feeding?

Diagram I. would seem to have answered this question affirmatively. Before, however, stating this definitely to be the case, it is advisable to consider whether some other factor may not be concerned, to which this difference can be attributed.

Such a factor might be the error due to the so-called "Error of Sampling." If this error is significant, then the curves may have a different interpretation to the apparently obvious one, and it therefore becomes essential to examine the importance of this factor, before proceeding to draw deductions from the curves as they stand in Diagram I.

*Analysis of the Data by Statistical Methods.**—In dealing with the error of sampling the important point will evidently be to ascertain how much the mean value obtained from the observations as shown on the curves is likely to differ from the mean of all babies in the same class, that is to say what is the probable error of the mean.

Suppose M_1 and M_2 are the means of the two sets of observations, then the accuracy of each must evidently depend upon

(*a*) The number of observations upon which it is based, and
(*b*) The divergence of these observations from their mean value.

In statistical work the expression $\cdot 67449 \frac{s}{\sqrt{N}}$ is taken to represent the probable error, where s = the square-root of the average of the squares of the distances of the observations from the mean, and is known as the "Standard Deviation," and where N = the number of observations. (Cp. Yule. Introduction to the Theory of Statistics. Chaps. VII. and XVII.)

The measures of reliability or the "probable errors" for the two means will be $\cdot 67449 \frac{s_1}{\sqrt{N_1}}$ and $\cdot 67449 \frac{s_2}{\sqrt{N_2}}$ respectively. These expressions may be called E_1 and E_2.

Experience has shown that unless the difference between M_1 and M_2 is at least two or three times as great as $\sqrt{E_1^2 + E_2^2}$ then it is not safe to assert that the difference found is really significant; it might be due to an error of sampling.

This method is only strictly speaking applicable when the variables, *i.e.*, the observations are "normally" distributed (vide Yule, op. cit. Chap X.) but it may fairly be used as a sufficiently accurate test for material such as the present.

This test of the error of sampling has been applied over three periods of eight days, in each of the series. The three period selected were the three consecutive periods included from the 137th to the 160th day after birth. These periods were selected as being those where there were a large number of observations in both series, and where the numbers of each series were most nearly equal.

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* For instruction in the statistical methods employed and for supervision of the results obtained I am deeply indebted to Dr. Major Greenwood, Junr., of the Lister Institute, and have much pleasure in thanking him for his most valuable help.

The unit of grouping taken was 200 grammes, and the results obtained are given in the accompanying table.

Days of age.	Mean weight (in grammes).		Difference $M_1 \sim M_2$ (in grammes).	Probable error.		Value of $\sqrt{E_1^2+E_2^2}$.	Ratio of $\frac{M_1 \sim M_2}{\sqrt{E_1^1+E_2^2}}$.
	Series I.	Series II.		Series I.	Series II.		
137–144	5,929	5,436	495	44·98	44·59	63·6	7·8
145–152	6,033	5,569	455	43·84	41·20	60·1	7·6
153–160	6,237	5,669	548	48·25	44·00	65·1	8·4

The mean of these observations bears therefore such a ratio to the value of $\sqrt{E_1^2+E_2^2}$ as to show clearly that the difference between the mean values of the two series can hardly be due to an error of sampling.

It appears that there is a difference between the values obtained for the series of babies fed upon the breast and for those fed upon boiled cows' milk, and that this difference can hardly be attributed to errors of sampling. It does not, however, necessarily follow that the difference of food has been the causative factor, and it becomes necessary to ask whether there can be any other factor at work which is producing the difference found.

The question of the health of the children need not be taken into consideration since the children were all, as far as it was possible to judge from the careful medical notes, in a good state of health.

The social class of the children seemed a possible factor, and in spite of the fact that the parents of the children all belonged approximately to the same social class, it was considered advisable to investigate the possible significance of any difference which existed between the social conditions of the homes.

Nothing very striking in this direction could be expected at the outset, since in no case was the family in circumstances of more than moderate comfort, such persons not being encouraged to attend the consultation, and only being received in quite exceptional cases. On the other hand no infant was allowed to suffer from any deficiency of food, since in cases of poverty the milk for the infant is supplied free by the town. Hence the question of possible relative starvation does not come in at all.

In order to arrive at the significance, if any, of the social conditions upon the observed difference in the weight of the two series of babies, it was necessary to investigate the relation between the weight of the infant, the wages of the father, and the method of feeding.

For this purpose the weights of the babies of both series at the age of 137–144 days of life were taken. Illegitimate infants and those of families who were living upon the wages of the mother, were omitted, as not being entirely comparable. 345 observations were available for this purpose.

The correlations between the three variables taken in pairs were found to be—

*(1) For artificial feeding and wages, ·16.
*(2) For artificial feeding and weight of child, – ·28.
†(3) For weight of child and wages of father, – ·02.

The correlation between weight of the child and the method of feeding was determined, the wages of the father being kept constant, (coefficient of partial correlation, *vide* Yule, *op. cit.*, pp. 225–249) and the figure obtained was – ·29 ± ·03, and the correlation between wages of parents and weight of the infant, keeping the feeding constant, is – ·026 ± ·036.

It would appear therefore that the weight of the child is more closely associated with the nature of the food than with the social class, as indicated by the wages of the father.

It must, however, again be emphasised that we are here dealing with a class of persons who are by no means a random sample of the population, but are almost a selected social class. A table of the wages of the parents as found to be distributed in making out the correlation of the feeding in relation to the wages is given below (see Table VI); it might appear at first sight that this table shows a considerable range of distribution since the wages show a variation of from 10–42 marks a week. A further examination will show that the great majority of the fathers were earning from 20–30 marks a week, that is to say, they were earning the average artisan wage, and the cases taken belong as a whole to a definite class of the population; it would be hardly possible to confine the cases to a smaller range of wages than 20–30 marks a week. The cases falling below this figure are rendered more equal than appears from the table by the fact that no baby is allowed to have a deficient food-supply, since if it is artificially-fed the milk is supplied free if the family cannot afford to pay for it, and the nursing mothers receive a nursing bonus, and if necessary may receive a further subsidy.

The question of environment may have some influence in this connection since presumably a family earning 15 marks a week will not live in such sanitary rooms as one earning 30 marks, but this is again partly mitigated by the regular visits of the health visitor, who endeavours to procure fresh air and cleanliness for the child.

Hence these figures, although of interest, cannot be used for more general deductions as to the relation of the weight, wages, and method of feeding of the infant population as a whole. It is fairly certain that, inasmuch as few of the more prosperous artisans attend the consultation the correlation between weight of infant and wages is less than would have been found in the general population. Even, however, assuming that the infants attending the consultation belonged to much more widely distributed social strata, it is unlikely that the correlation would be raised to any value approaching that found for the correlation between the method of feeding and the body-weight. Moreover this last correlation would probably be higher in the general population.

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* Determined by the method of Pearson (3) (in Biometrika VII., 1909, p 96).

† Determined by the ordinary product moment method. (Cp. Yule ([illegible]), *op. cit.*, Chap. IX.)

The –ve sign indicates that body-weight decreases as the proportion of artificially-fed babies increases.

The distribution of the weight in relation to the food of the infants under consideration is given in Table V and shows that the maximum of the weight-frequency occurs at a lower level of weight in the boiled cows' milk series than in the breast milk series. Table VI gives the distribution of the feeding in relation to wages.

In both Tables V and VI there are only 345 observations in a total number of 504 children owing almost entirely to the fact that a number of the children did not happen to come up for weighing at the age of life which was taken, namely, from 137–144th day of life. Only a very few children were omitted because of illegitimacy or because the mother was the wage-earner of the family.

TABLE V.

Showing the distribution of the Observations on the Weight of Infants in relation to their Food at the 137–144*th day of life.*

Weight of Infants in grammes.	Number of Infants having such a weight		
	In breast milk series.	In boiled milk series.	Total.
3000–3200	1·0	·5	1·5
3200–3400	1·0	·5	1·5
3400–3600	0	0	0
3600–3800	4·0	2·5	6·5
3800–4000	3·0	3·5	6·5
4000–4200	6·0	3·5	9·5
4200–4400	3·0	6·0	9·0
4400–4600	2·0	4·0	6·0
4600–4800	6·5	8·5	15·0
4800–5000	8·0	9·5	17·5
5000–5200	10·0	11·0	21·0
5200–5400	10·0	10·5	20·5
5400–5600	13·5	10·5	24·0
5600–5800	14·0	17·5	31·5
5800–6000	16·5	16·0	32·5
6000–6200	19·0	15·5	34·5
6200–6400	20·5	4·5	25·0
6400–6600	11·0	7·0	18·0
6600–6800	18·0	3·0	21·0
6800–7000	10·0	4·0	14·0
7000–7200	13·5	·5	14·0
7200–7400	6·5	·5	7·0
7400–7600	—	·5	·5
7600–7800	3·0	·5	3·5
7800–8000	2·5	—	2·5
8000–8200	·5	—	·5
8200–8400	1·5	—	1·5
8400–8600	·5	—	·5
	205·0	140·0	345·0

TABLE VI.

Showing the distribution of the Observations on the Feeding of the Infants in relation to the Wages.

Weekly Wages of Father in Marks.	Number of Fathers earning such weekly wage		
	In breast milk series.	In boiled milk series.	Total.
10	1·0	·5	1·5
11	4·0	2·5	6·5
12	0·0	0·0	0·0
13	·5	·5	1·0
14	·5	·5	1·0
15	3·0	0·0	3·0
16	·5	·5	1·0
17	2·0	1·0	3·0
18	2·5	2·5	5·0
19	7·0	2·0	9·0
20	22·5	13·0	35·5
21	20·5	7·0	27·0
22	31·0	15·5	46·5
23	28·0	12·5	40·5
24	17·0	14·0	31·0
25	20·5	19·5	40·0
26	8·0	5·0	13·0
27	11·0	12·0	23·0
28	9·5	9·5	19·0
29	1·5	5·0	6·5
30	10·0	13·0	23·0
31	2·0	0·0	2·0
32	·5	0·0	·5
33	·5	1·0	1·5
34	—	—	0·0
35	—	2·0	2·0
40	1·0	1·0	2·0
42	1·0	—	1·0
	205·0	140·0	345·0

The following inferences may be drawn as to the divergence the two curves in Diagram I up to the 208th day :—

(1) There is a significant difference between the average weight of infants fed upon the breast and upon boiled cows' milk, in favour of the former ; and

(2) An important factor in this result is the method of feeding.

The curves of Diagram I may be divided into three parts, namely :—

(1) The first part where the curves cross and then diverge ; the curve of the boiled milk series, which starts above the curve of the breast-fed series, falling rapidly below this latter curve.

(2) The second part of the curves where the two curves run approximately parallel from about the 24th to the 200th day of life, and

(3) The last part of the curves where the divergence is obliterated, the subsequent tendency being for the curve of the boiled cows' milk series to show a value a little above that of the breast-fed series.

From the preceding statistical analysis it appears that the divergence of the *middle part* of the curve is to be attributed essentially to the difference in the method of feeding of the two series.

The first and third parts of the curves remain for consideration and will now be dealt with, the first part being taken first.

Further Analysis of the First Part of the Curves of Diagram I.—At no part of the curves is the tendency to diverge so markedly shown as in the first part of the curves, during a period extending over the first three of the eight-day periods of life. The average weight of the breast-fed babies shows a rise from the first, while that of the babies fed upon boiled cows' milk falls throughout the two first eight-day periods, and shows no rise above the first eight-day period until the 33–40th days of life.

It is a matter of common knowledge that every baby loses weight during the first few days of life, and a drop in the average weight of the breast-fed babies in the second eight-day period was almost to be expected. This possible fall in the curve is concealed to some extent by the grouping of the weights into periods of eight days, the first period including the period of fall in weight. In many of these cases the observations would commence at a time when the loss of weight after birth had already taken place, and the child was again beginning to increase in weight. The absence of fall in the curve of the breast-fed babies can therefore be explained.

When a comparison is made between the two curves, it appears that while one curve rises the other falls, and evidently there is either some fundamental factor or factors at work producing this difference, or some source of error has crept into one or both of the curves.

It was considered desirable first to eliminate any possible source of error. The same source of error as was sought for in the middle part of the curves may evidently be at work in this part of the curves, namely, the error of sampling, and this was therefore investigated.

Statistical Examination of the Average Weights obtained in the first four periods of Eight Days.

The same method and notation as were used in dealing with the middle part of the curves was applied, viz. :—

N = Number of observations.

s = Standard deviation.

E = Probable error, and is represented by the expression

$$\cdot 67449 \frac{s}{\sqrt{N}}$$

M_1 and M_2 are the means of the two series, their difference being "D."

By this method the following values were obtained and a tabulated below :—

—	Days of Age.	Mean (in grammes).	Standard Deviation.	Probable Error.	$\sqrt{E_1^2+E_2^2}$	$\frac{D}{\sqrt{E_1^2+E_2^2}}$
Series I ...	1–8	3,185	622	85·8		
		D=330			122·0	2·7
Series II ...	1–8	3,515	410	87·4		
Series I ...	9–16	3,312	544	37·3		
		D=222			60·5	3·7
Series II ...	9–16	3,090	452	48·3		
Series I ...	17–24	3,512	632	35·4		
		D=145			49·5	2·92
Series II ...	17–24	3 367	460	35·7		
Series I ...	25–32	3,745	652	34·1		
		D=272			47·5	5·7
Series II ...	25–32	3,473	522	33·1		

The average weight of the babies fed upon boiled cows' milk is higher for the first eight-day period than that of the breast-fed babies. The former value is based upon 10 observations, and the latter upon 24 ; it becomes a question whether any importance can be attributed to this difference in average weight or whether it may not be due to an error introduced by the extremely small number of observations available for the boiled cows' milk series.

Ten observations are not sufficient for the formula given in the above table of results to be justifiably employed, since the reliability of the method is exaggerated when the number of observations is very small.

The method introduced by "Student" (61) is applicable for small number of observations. It is based upon the probability of the occurrence of the mean value obtained by the ordinary method among the average population.

Taking 3185 ± 85·8 (the "probable error" of 3185 is 85·8) grammes as the mean weight of babies in the average population it appears that the chance of 10 observations from such a population having a mean of 3515 grammes with a standard deviation of 410 is 1 in 50. Suppose, however, that the mean weight of the average baby in the population were 3357 grammes, it is then found that the probability that a population with a mean weight of the babies of this age of 3357 grammes (3185 + twice the probable error, *i.e.*, 172) should give in 10 observations a mean of 3515 is 1 in 7. It may be remarked that so far as the evidence goes, there is about 1 chance in 10 that the mean weight of the controls is not less than 3359.

It seems therefore that the difference between the weights of the two series for the first eight-day period, might be considered as due to an error of sampling brought about by the extremely small number of observations available for the series of babies fed upon boiled cows' milk. It may be taken that the babies of both series whose weights were observed during this period of life can be considered as average samples of the population, the influence of other factors, if present, which would tend to cause a divergence of the two series, being inappreciable compared with that caused by the error of sampling.

The figures of the later periods, are based upon sufficiently large number of observations for the ordinary method to be reliable.

The tabulated results show that the ratio of the difference of the means to the measure of the sum or difference of the probable errors ($\sqrt{E_1^2 + E_2^2}$) is in all cases greater than 2, and hence the difference in weight of the two series, may fairly be attributed to some factor other than the error of sampling.

A source of error might arise in respect of the distribution of the variables.

In applying the usual method, it is assumed that these are "normally" distributed; inspection of the distribution of the individual weights suggests that this condition is not accurately fulfilled, and the process is not then strictly reliable ([34]).

The figures, however, approximate sufficiently to the normal type for it to be unlikely that an appreciable error is introduced in basing the results obtained upon the application of this formula.

Some factor other than the error of sampling must therefore be sought for.

The possible influence of the social conditions has already been dealt with fully in a previous section of this report (see pp. 41, 42) in connection with the middle part of the curves, and it has been shown that in this part of the population, which is to a great extent a selected population, this is a negligible factor. It need not therefore be raised again.

The main factor for consideration will evidently be that of the feeding and it seems not unreasonable to suppose that the loss in weight which occurs in all children is on the average more prolonged in babies fed upon boiled cows' milk, than in babies fed upon the breast.

Birk ([5]), in some experiments carried out upon the metabolism of infants in the first days of life, found that the breast-fed babies he investigated had regained their birth-weight in 4 or 5 days after birth. And it appears probable from the data given above in Table III. that the average breast-fed baby regains its birth-weight at any rate within a period of eight days, since there is a marked rise in average weight during the second eight-day period.

Birk found that although an infant may show absolute loss of weight during the first few days of life, yet it is retaining nitrogen, and in the cases studied by him, at the time the child regained its birth-weight, namely at the fifth day, there was a positive nitrogen balance of 951·3 milligrammes.

This, however, only occurred with breast-fed babies who were given colostrum.

A child fed upon the milk of a wet-nurse who was in a later stage of lactation, and had no longer any colostrum, showed a negative nitrogen balance. It may well be that the colostrum is of great importance in the metabolism of infants during the first days of life. Colostrum is very rich in nitrogen, and according to Pröscher ([57]) the nitrogen content of the milk has an important relationship to the rate of growth of the young animal of many species. Griffith and Gittings ([35]) found that the loss of weight after birth could be prevented, by giving the child to a wet-nurse during the first day or two, but that after a few weeks no difference could be detected between the infant whose loss of weight had been prevented, and one whose loss of weight had been allowed to occur.

The physiological loss of weight which occurs after birth appears to be greatly accentuated in the case of children fed upon boiled cows' milk, and it seems possible that the absence of colostrum is an important factor in its causation, in addition to the disadvantage arising from the use of the milk of another species.

No data appear to be available in regard to babies fed upon the colostrum of cows, either boiled or raw ; nor any upon infants fed upon raw cows' milk either in comparison with breast feeding or with boiled cows' milk.

There are a fair number of data in regard to the causation of the loss of weight in breast-fed babies, but this is scarcely within the scope of the present report.

The difference between the curves of the two series is in all probability very intimately connected with, if not entirely due to the method of feeding, and points to the great importance of breast-feeding for infants, especially in the early days of life : this is also in entire accord with the clinical experience and has been forcibly voiced by Czerny ([20]) and many others.

Analysis of the latter parts of the curves.—The difference between the average weights of the two series begins to decrease at about the 180th day of life, and then disappears fairly rapidly, until at about the 230th day it is no longer present. From this age onward no difference between the two curves can be detected, except possibly in favour of the boiled milk series towards the end of the first year.

If Tables III. and IV. be referred to it will be seen that the number of observations is becoming smaller, and that therefore the reliability is somewhat lessened ; the numbers are however still sufficiently large for further statistical investigation to be unnecessary.

A considerable number of the healthy babies of the breast-fed series, which had been weaned at about the age of nine months, ceased attending before the end of the first year, and there is almost certainly a tendency for the less robust breast-fed babies to predominate, in this series. The babies fed upon boiled cows' milk tend on the whole to be brought up to the consultation to a somewhat later age, partly no doubt because the mother having got accustomed to getting the milk through the consultation, and knowing it to be reliable, prefers to continue getting the milk in this manner, and partly also because, if she is poor, she gets the milk at a reduced rate.

It was stated (on p. 35) that of the 300 babies of the breast-fed series, 52 were weaned between the ages of four and six months, so that a not inappreciable number of the babies of Series I. were actually receiving the same food as the babies of the other series, during the period from the 180th day onwards.

It is not improbable also, that many of the mothers who were still feeding their infants during the period of from 6–9 months, were not giving such a free supply of milk for the infant as at an earlier period of the baby's life. It might well be that the infant was receiving sufficient milk for the needs of the organism, but not as much as was required for an optimum rate of growth, and might not be obtaining as rich a food-supply as the babies of the boiled cows' milk series, who were receiving a carefully regulated quantity of food material.

It appears from the work of Schlossmann ([59]), Budin ([12]), and Finkelstein ([30]) that many wet-nurses are capable of giving large quantities of milk over prolonged periods, amounting in some cases to 10–13 months. This is, however, not universal. There is the additional consideration that the wet-nurses are presumably in much more favorable circumstances in the regular life and food of an institution, than the mothers of the average artisan family.

In Schlossmann's cases it was found that the quantity of milk given by a woman who was considered an excellent wet-nurse, and was under very favorable circumstances increased up to about the 60–70th day of lactation, and then maintained a fairly constant and high level, up to about the 200–210th day of lactation, after which it became irregular in quantity and tended to decrease. He observed the same also in a second case.

Budin and Finkelstein's cases show a longer period of full lactation in most of the wet-nurses.

Bunge ([14]) states that the period of lactation in women is gradually becoming shorter. Without entering into this much discussed question, it may be mentioned that the age of weaning is now considerably younger than it was some time ago, before the introduction of artificial feeding. (Cp. Introduction to this report p. 2.) It may be that the optimum length of breast-feeding in the human species is the same as it always was, but that children were formerly kept upon the breast for a period exceeding the physiological period, and a high degree of under-feeding may have been the result : the extremely high rate of mortality among young children in the middle ages and later, may have had some connection with an unduly prolonged lactation.

In this connection Silbergleit ([60]) has recently shown that among the population of Berlin as a whole, the percentage of breast-fed infants has decreased during the ten years from 1895 to 1905, and that the percentage of bottle-fed infants has increased disproportionately for the later months of infancy. In other cases the duration of breast-feeding is becoming shorter among the population of Berlin as a whole.

It does not seem necessary to seek any further for reasons as to the causes of the disappearance of the difference between the average weights of the two series.

Under the conditions such as obtained in the consultation from whose records the observations dealt with in this report have been compiled, it seems that after the age of six to eight months no appreciable difference in weight between the breast-fed babies and babies fed upon boiled cow's milk can be detected.

It is impossible to say how far this would hold for the population at large, since as soon as the babies come under observation in the matter of weight, they likewise come under medical care and attention ; there can however be very little doubt, that this favorable result is due in a great measure to the medical care and knowledge which is available for the babies attending the Consultation.

If the average weights of the babies of both series are compared with the corresponding figures given by Camerer (17), it appears briefly that, neglecting the weight at birth, the rate of growth of Berlin babies given in this report is rather less than that given by Camerer. As a whole, the initial weights are higher and the final weight at the end of the first year, lower.

Compared with the figures of Finkelstein (Waisensäuglinge. p. 9) who has compared his figures with those of Camerer, it seems that the figures given in this report are intermediate in value between the average values obtained by Camerer and Finkelstein. Owing to the differences between the quantity and quality of the material used in the different sets of observations it would not be profitable to carry the comparison any further.

The percentage rate of growth of babies of both series.—It seemed desirable to study the rate of growth of the babies of the two series dealt with in this report.

This was done in the following ways :—

1. By estimating the percentage increase per kilo. of body-weight during each period of eight days, each series being taken separately.
2. The rate at which growth took place as measured by the time required by the babies of each series for the doubling of the initial average weight, the values of the first eight-day period being omitted on account of the small number of observations available in the series of babies fed upon boiled cows' milk.
3. By estimating the percentage deficit of the average weight of babies fed upon boiled cows' milk as compared with the weights of the breast-fed babies. The weights of the breast-fed babies being taken as 100.

The results of the first method are given in Table VII. and are plotted in Diagram II. Both series show an extreme irregularity in the rate at which the weight increases, but after the first two estimations it would be difficult to point out any marked difference between the values of the two series.

TABLE VII.

Showing percentage rote of increase for each period of eight-days, of the babies of both series, up to the age of eight months.

Age in days.	Breast-fed Series.			Boiled Cows' Milk Series.		
	Average Weight (in grammes).	Increase.		Average Weight (in grammes).	Increase.	
		In grammes.	Per cent.		In grammes.	Per cent.
1–8	3185	—	—	3515	—	—
9–16	3348	163	5·1	3090	—425	—12·0
17–24	3507	159	4·7	3358	268	8·7
25–32	3746	239	6·8	3472	114	3·4
33–40	3939	193	5·1	3708	236	6·8
41–48	4119	180	4·6	3848	140	3·8
49–56	4291	172	4·2	3991	143	4·1
57–64	4443	152	3·5	4082	91	2·9
65–72	4638	195	4·4	4240	158	3·9
73–80	4737	99	2·1	4407	167	3·9
81–88	4937	200	4·2	4486	79	1·8
89–96	5079	142	4·9	4628	142	3·2
97–104	5191	112	2·2	4814	186	4·0
105–112	5380	189	3·6	4935	121	2·5
113–120	5566	183	3·6	5052	117	2·8
121–128	5659	93	1·7	5171	119	2·4
129–136	5757	98	1·7	5326	155	3·0
137–144	5929	172	3·0	5436	110	2·0
145 152	6033	104	1·8	5569	133	2·4
153–160	6237	204	3·4	5669	100	1·8
161–168	6274	37	·6	5831	162	2·9
169–176	6312	38	·6	5915	84	1·4
177–184	6434	122	1·9	6146	231	3·9
185–192	6458	24	·4	6242	96	1·6
193–200	6664	206	3·2	6319	77	1·2
201–208	6709	45	·7	6475	156	2·5
209–216	6734	25	·4	6467	— 8	— ·1
217–224	6798	64	1·0	6677	210	3·2
225–232	6778	— 20	— ·3	6721	44	·7
232–240	6886	108	1·6	6770	49	·7

After this age the increase becomes very variable.

The results of the second method are shown in Table VIII. and are plotted on Diagram III., which shows that as regards rate of growth there is little to choose between the two series. The babies fed upon boiled cow's milk doubled their weight at the 185–192nd day, or a fortnight earlier than the breast-fed babies, but they started with an initially lower weight, since the first value was omitted, on account of the small number of observations.

TABLE VIII.

Showing the percentage rate of Growth of the Babies of both series starting from the second eight-day Period of Life. Control series being the Breast-fed Babies and the other series being Babies which were fed upon Boiled Cows' Milk.

Age in days.	Control Series.			Boiled Milk Series.		
	Average Weight (in grammes).	Increase.		Average Weight (in grammes).	Increase.	
		In grammes.	Per cent		In grammes.	Per cent.
8–16	3348	—	—	3090	—	—
17–24	3507	159	4·7	3358	268	8·6
25–32	3746	398	11·8	3472	382	12·3
33–40	3939	591	17·6	3708	618	20·0
41–48	4119	771	23·0	3848	758	24·5
49–56	4291	943	28·1	3991	901	29·1
57–64	4443	1095	32·7	4082	992	32·1
65–72	4638	1290	38·5	4240	1150	37·2
73–80	4737	1389	41·4	4407	1317	42·6
81–88	4937	1589	47·4	4486	1396	45·1
89–96	5079	1731	51·7	4628	1538	49·7
97–104	5191	1843	55·0	4814	1724	55·7
105–112	5380	2032	60·6	4935	1845	59·7
113–120	5566	2218	66·2	5052	1962	63·4
121–128	5659	2311	69·0	5171	2081	67·3
129–136	5757	2409	71·9	5326	2236	72·3
137–144	5929	2581	77·0	5436	2346	75·9
145–152	6033	2685	80·1	5569	2479	80·2
153–160	6237	2889	86·2	5669	2579	83·4
161–168	6274	2926	87·3	5831	2741	88·7
169–176	6312	2964	88·5	5915	2825	91·4
177–184	6434	3086	92·1	6146	3056	98·8
185–192	6458	3110	92·8	6242	3152	102·0
193–200	6664	3316	99·0	6319	3229	104·4
201–208	6709	3361	100·3	6475	3385	109·5
209–216	6734	3386	101·1	6467	3377	109·2
217–224	6798	3450	103·0	6677	3587	116·0
225–232	6778	3430	102·4	6721	3631	117·5
233–240	6886	3538	102·6	6770	3680	119·0

The third method, giving the percentage deficit of the average weights of the babies fed upon boiled cows' milk as compared with the breast-fed babies, shows that at no period of life does the deficit exceed 10 per cent., and that during the greater part of the period under observation the deficit is much less (see Table IX.).

It is probable that this favourable result is largely due to the fact that these babies were attending the consultation, and were therefore under favorable conditions. It is a more favorable result than the majority of the results that have been obtained in the feeding experiments upon animals who received the milk of a foreign species. The results of clinical experience and the evidence given in Parts II. and III. of this report, render it doubtful whether this result could be surpassed by feeding babies upon raw cows' milk.

TABLE IX.

Showing the percentage relationship between the average weights of the babies of the two series.

Series I. consisting of breast-fed babies, and Series II. consisting of babies fed upon boiled cows' milk.

Age in days.	Average weight in grammes.		Difference (in grammes).	Percentage value of Series II to Series I.
	Series I.	Series II.		
1–8	3,185	3,515	—330	110·3
9–16	3,348	3,090	258	92·2
17–24	3,507	3,358	149	95·7
25–32	3,746	3,472	274	92·6
33–40	3,939	3,708	231	94·1
41–48	4,119	3,848	271	93·4
49–56	4,291	3,991	300	93·0
57–64	4,443	4,082	361	91·8
65–72	4,638	4,240	408	91·4
73–80	4,737	4,407	330	93·0
81–88	4,937	4,486	451	90·8
89–96	5,079	4,628	451	91·1
97–104	5,191	4,814	377	92·7
105–112	5,380	4,935	445	91·7
113–120	5,566	5,052	514	90·7
121–128	5,659	5,171	488	91·3
129–136	5,757	5,326	431	92·5
137–144	5,929	5,436	493	91·6
145–152	6,033	5,569	464	92·3
153–160	6,237	5,569	568	90·8
161–168	6,274	5,831	443	92·9
169–176	6,312	5,915	397	93·7
177–184	6,434	6,146	288	95·5
185–192	6,458	6,242	216	96·6
193–200	6,664	6,319	345	94·8
201–208	6,709	6,475	234	96·5
209–216	6,734	6,467	267	96·0
217–224	6,798	6,677	121	98·2
225–232	6,778	6,721	57	99·1
233–240	6,886	6,770	116	98·3
241–248	6,891	7,010	—119	101·7
249–256	7,118	7,112	6	99·9
257–264	7,276	7,204	72	99·0
265–272	7,217	7,274	—57	100·7

Note.—After this age the percentages are very variable.

Further analysis of a portion of the material.—It seemed desirable to investigate the average weights of the babies whose weight-charts and notes had been copied for the purposes of this report, in order to get two series, one consisting of babies exclusively breast-fed, and the other consisting solely of babies who had never been breast-fed, but who had been fed from birth upon boiled cows' milk only.

The charts were re-sorted, and it was found that among the babies of the breast-fed series, there were 130 whose records were available from about a fortnight after birth up to at least the 200th day of life, and who during this period had received only breast milk.

Among the 204 babies who had been fed upon boiled cows' milk there were 78 who had attended the consultation during the same period of life and who had received only boiled cows' milk. In addition there were 41 babies who had similar attendance records, and who had been fed for periods of not more than eight days upon the breast before receiving boiled cows' milk, as their sole food.

In order to ascertain whether these 41 babies could reasonably be included in one series with the babies who had never been breast-fed, the average weights for the 78 babies who had never been breast-fed and the average weights for those 41 babies who had received the breast for these few days, were worked out separately. No appreciable difference in the average weights was found, so the two sets of babies were added together, and worked up as a series of 119 babies who had been fed exclusively upon boiled milk. The figures for the 78 and 41 babies respectively are given in Table X.

TABLE X.

Showing the average weights and the number of estimations of 78 babies who were never breast-fed, and of 41 babies who were breast-fed for eight days or less.

Age in days.	78 babies never breast-fed.		41 babies breast-fed 1–8 days.	
	Average weight.	Estimations.	Average weight.	Estimations.
1–8	3,350	3	4,325	2 (same baby)
9–16	3,020	17	3,200	9
17–24	3,350	39	3,261	20
25–32	3,485	56	3,245	26
33–40	3,686	55	3,225	35
41–48	3,770	59	3,668	35
49–56	3,888	57	3,906	36
57–64	3,995	68	3,781	34
65–72	4,095	66	4,198	33
73–80	4,254	67	4,348	32
81–88	4,424	86	4,342	40
89–96	4,555	81	4,543	29
97–104	4,769	72	4,787	39
105–112	4,810	65	4,757	38
113–120	4,985	66	4,820	41
121–128	5,073	63	5,020	41
129–136	5,314	68	5,234	33
137–144	5,393	58	5,132	41
145–152	5,483	56	5,526	41
153–160	5,551	58	5,573	31
161–168	5,427	60	5,748	34
169–176	5,816	63	5,803	34
177-184	6,025	49	5,959	33
185–192	5,861	64	5,941	26
193–200	6,295	50	6,335	31

The figures obtained by estimating the average weights of the 130 exclusively breast-fed babies and of the babies fed exclusively upon boiled cows' milk, show the same main characteristics as the curves of the two original series of babies the results of which series were plotted in Diagram I. The divergence is somewhat more accentuated, but the tendency for the two curves to approach one another after about the 200th day, begins to appear after about the 160th day.

The number of observations for the first eight-day period are too small in the case of the babies fed upon boiled cows' milk to merit any special consideration. The average weights are given on Table XI. and the results are plotted in Diagram IV.

TABLE XI.

Showing the average weight of 130 *Babies exclusively breast-fed up to* 200 *days of age, and of* 119 *Babies of whom* 78 *had never been breast-fed, and of* 41 *who were breast-fed for less than eight days.*

Age in days.	Breast-fed babies.		Babies fed on boiled milk.	
	Average weight (in grammes).	No. of observations.	Average weight (in grammes).	No. of observations.
1–8	3,019	13	3,740	5
9–16	3,325	42	3,083	26
17–24	3,499	71	3,320	59
25–32	3,738	91	3,409	82
33–40	3,955	87	3,682	90
41–48	4,171	103	3,732	94
49–56	4,327	85	3,895	93
57–64	4,506	107	3,923	102
65–72	4,760	103	4,129	99
73–80	4,836	99	4,284	99
81–88	5,102	102	4,398	126
89–96	5,153	98	4,552	110
97–104	5,360	91	4,775	111
105–112	5,530	95	4,791	103
113–120	5,688	98	5,016	105
121–128	5,808	95	5,052	104
129–136	5,020	82	5,288	101
137–144	6,085	97	5,285	99
145–152	6,229	84	5,501	97
153–160	6,272	81	5,559	89
161–168	6,294	67	5,716	94
169–176	6,381	84	5,811	97
177–184	6,597	71	5,999	82
185–192	6,620	67	5,884	90
193–200	6,856	64	6,310	81

The *percentage rate of growth* of the babies of these two series was calculated up to the day at which they doubled their weight, starting from the second eight-day period. Both the breast-fed babies and the babies fed upon boiled cows' milk doubled their weight at the 193–200th day of life. The rate of growth as shown by the figures giving the percentage increase is rather more rapid in the series of breast-fed babies, than in the series of babies fed upon boiled cows' milk. The figures are given in Table XII. and are plotted in Diagram V.

TABLE XII.

Showing the percentage rate of growth of the 119 *babies who had never been breast-fed, and of the* 130 *babies who were exclusively breast-fed, starting from the average weight at the end of the sixteenth day of age, and continuing up to the time at which they doubled their weights.*

Age in days.	Breast-fed Babies.			Babies never breast-fed.		
	Average Weight (in grammes).	Increase.		Average Weight (in grammes).	Increase.	
		In grammes.	Per cent.		In grammes.	Per cent
9–16	3,325	—	—	3,083	—	—
17–24	3,499	174	5·23	3,320	237	7·68
25–32	3,738	413	12·42	3,409	326	10·57
33–40	3,955	630	18 94	3,682	599	19·42
41–48	4,171	846	25·44	3,732	649	21·05
49–56	4,327	1 002	30·13	3,895	812	26·33
57–64	4,506	1,181	35·51	3,923	840	27·24
65–72	4,760	1,435	43·15	4,129	1,046	33·92
73–80	4,836	1,511	45·44	4,284	1,201	38·95
81–88	5 102	1,777	53·44	4,398	1,315	42·65
89–96	5,153	1,828	54·97	4,552	1,469	47·64
97–104	5,360	2.035	61·20	4,775	1 692	54·88
105–112	5,530	2,205	66·31	4,791	1,708	55·40
113–120	5,688	2,363	71·06	5,016	1,933	62·69
121–128	5,808	2,483	74·67	5,052	1.969	63·86
129–136	5 920	2,595	78·04	5,288	2,205	71·52
137–144	6,085	2,760	83·00	5,285	2 202	71·42
145–152	6,229	2,904	87·33	5 501	2,418	78·45
153–160	6,272	2,947	88·63	5,559	2,476	80·31
161–168	6,394	3,069	92·30	5,716	2,633	85·40
169–176	6,381	3,056	91·90	5,811	2,728	88·48
177–184	6,597	3,272	98·40	5,999	2 916	94·58
185–192	6 620	3,295	99·09	5 884	2,801	90·85
193–200	6,856	3,531	106·19	6.310	3,227	104·67

Summary of Results obtained in Part IV.—The Berlin figures dealt with in this part of the report show that, given circumstances similar to those of the Consultation from whence the figures are taken—

(1) Infants fed upon the breast show a higher average weight than infants fed upon boiled cow's milk, up to about the 180–220th day of life. After this age the difference in average weight disappears.

(2) This difference must be attributed to the method of feeding, and not to differences in the social condition of the infants.

(3) The difference in average weight is most marked in the first 16 days of life; this difference must be attributed to the different method of feeding, and not to any possible "error of sampling."

(4) After the first 16 days the average increase in body-weight is almost identical in both series of babies.

(5) The deficit of average weight of the babies fed upon boiled cows' milk below those fed upon the breast, does not reach 10 per cent. at any period.

PART V.—SUMMARY AND CONCLUSIONS.

The balance of evidence both experimental and clinical points in the main to the same conclusions. Both lines of research show—

(1) That there is apparently no serious loss of nutritive value produced by feeding an animal upon boiled milk derived from an animal of the same species. At the same time it must be pointed out that the published evidence on this point is scanty.

(2) That, when an animal is fed upon the milk of another species, the milk from which has been found to be suitable for this purpose, such small differences as have been found in the nutritive values of raw and boiled milk have been in favour of boiled milk.

(3) That the milk of the same species has a considerably higher nutritive value for that species than the milk of any other species so far investigated.

The evidence dealt with throughout this report emphasises very forcibly the importance of breast-feeding for the young of all species and shows the special importance of breast-feeding during the early weeks of life.

Where artificial feeding has been employed in animal experiments, boiled milk of a foreign species has given more satisfactory results than similar milk raw. The Berlin figures dealing with infants fed on boiled cows' milk, give extremely favourable results, and in view of the evidence collected in this report could scarcely be expected to be surpassed had raw cows' milk been used.

It may be again pointed out that the Berlin babies who are artificially-fed in connection with the consultation receive milk of a known excellent quality. The excellence of the results obtained in Berlin are almost certainly largely due to the care and supervision exercised at and through the consultation.

The foundations of the information given in this report were obtained by me, when working at the Lister Institute as Jenner Research Scholar, during which period I was also sent abroad to study the methods used in the countries of the Continent in the prevention of infantile mortality, especially in connection with the feeding of infants.

The Berlin material was obtained specially for this report to the Local Government Board.

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DIAGRAM I.

SHOWING THE AVERAGE WEIGHTS OF THE INFANTS OF SERIES I (CONTROL SERIES, CHIEFLY BREAST-FED) & OF THE INFANTS OF SERIES II (FED ON BOILED COWS' MILK) IN PERIODS OF EIGHT DAYS.

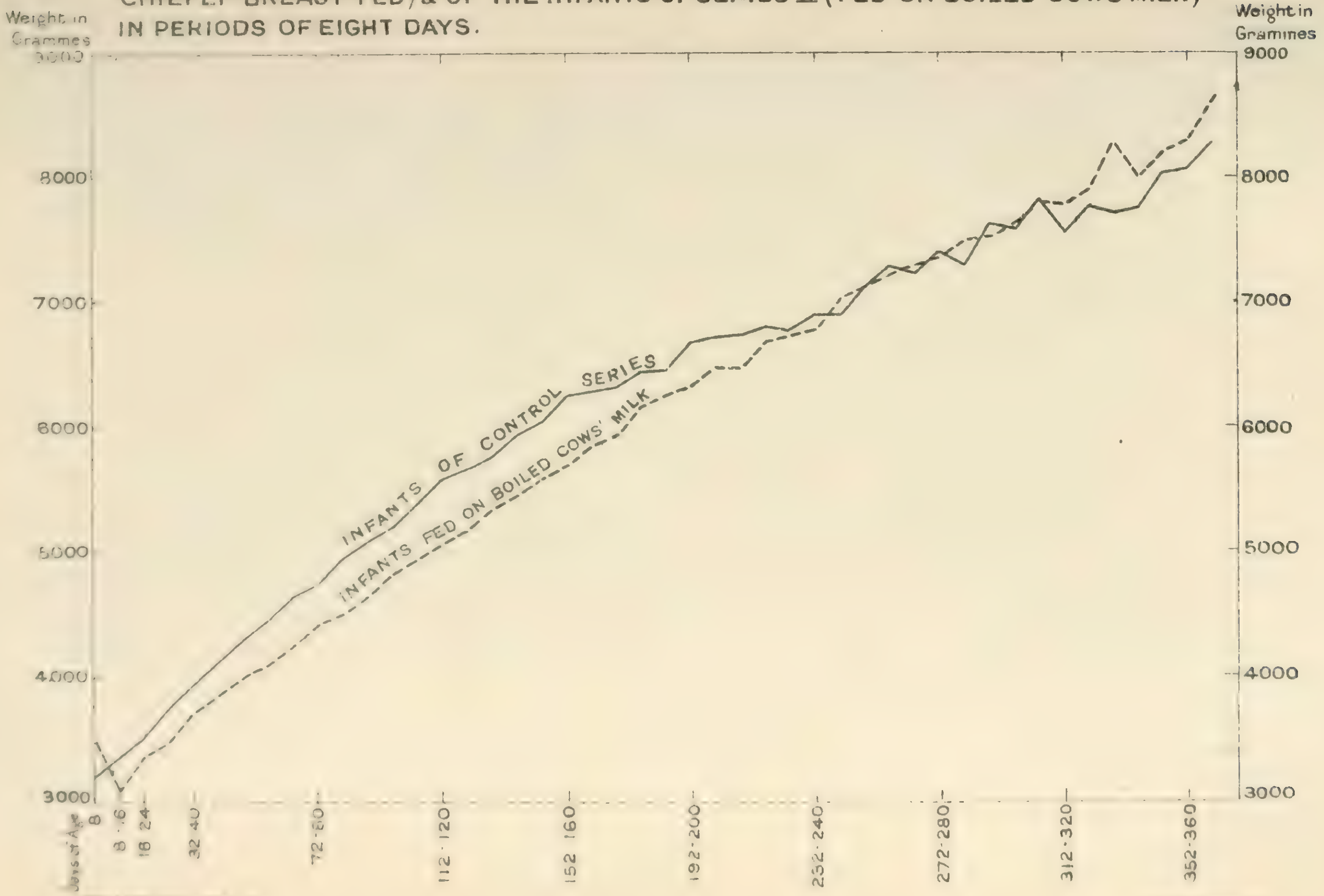

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DIAGRAM II.

SHOWING PERCENTAGE RATE OF INCREASE FOR EACH PERIOD OF EIGHT DAYS ON THE INFANTS OF BOTH SERIES (CONTROL & BOILED COWS MILK) UP TO THE AGE OF EIGHT MONTHS.

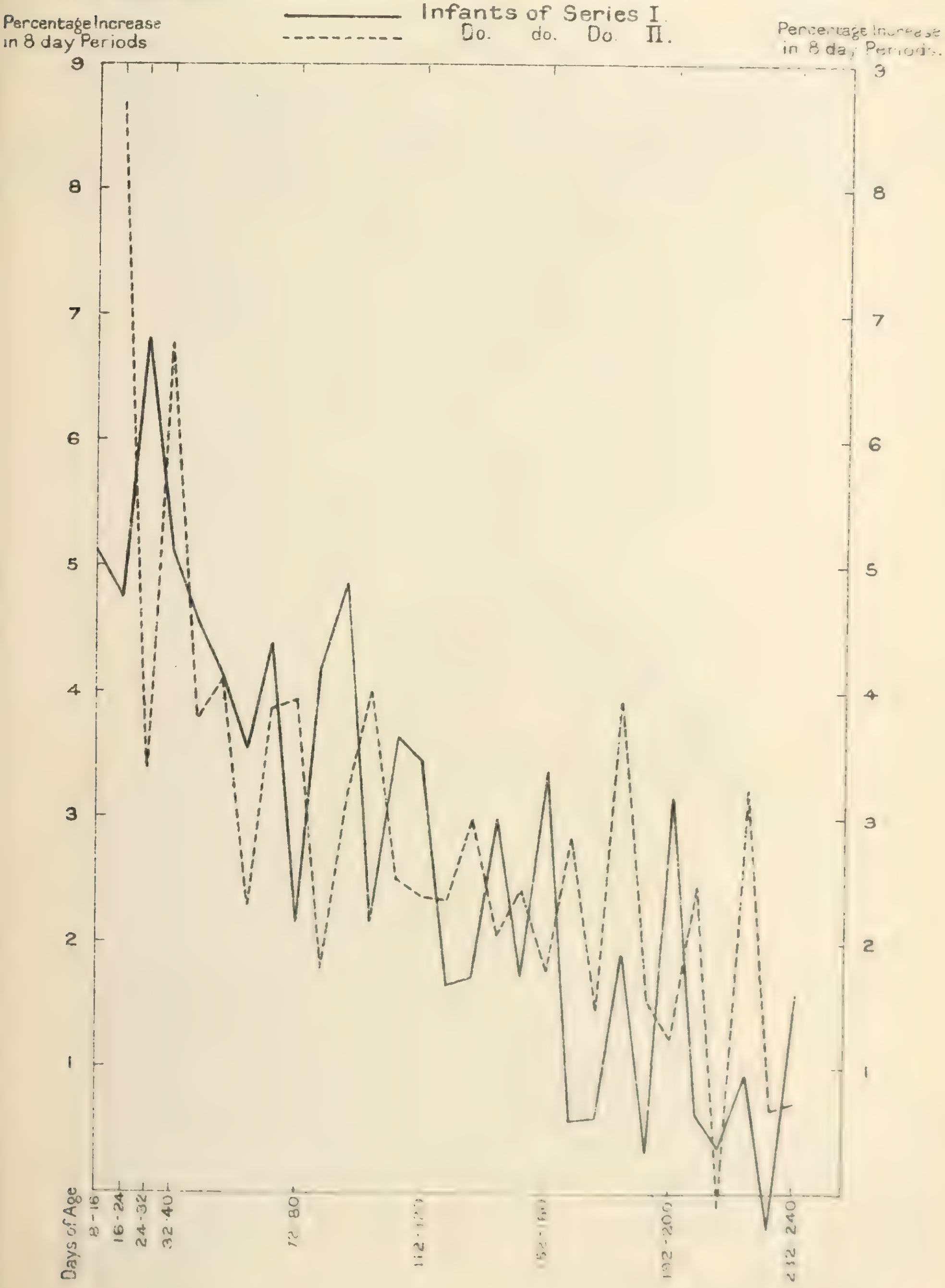

DIAGRAM III.

SHOWING THE PERCENTAGE RATE OF GROWTH OF THE INFANTS OF BOTH SERIES (CONTROL & BOILED COWS' MILK) COMMENCING AT THE SIXTEENTH DAY OF LIFE.

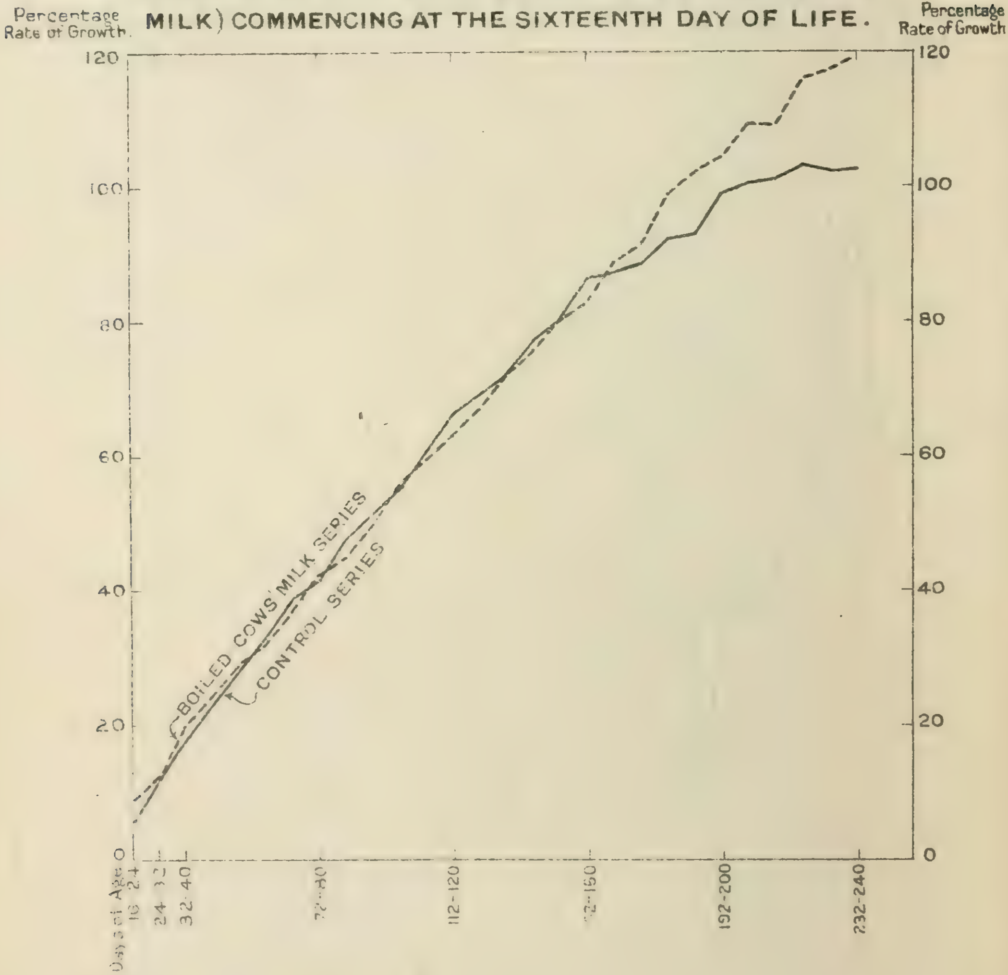

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DIAGRAM IV.

SHOWING THE AVERAGE WEIGHT OF 130 INFANTS EXCLUSIVELY BREAST-FED & OF 119 INFANTS EXCLUSIVELY FED UPON BOILED MILK UP TO 200 DAYS OF AGE. OF THE 119 INFANTS 78 HAD NEVER BEEN BREAST-FED AND 41 HAD BEEN BREAST-FED FOR 8 DAYS OR LESS.

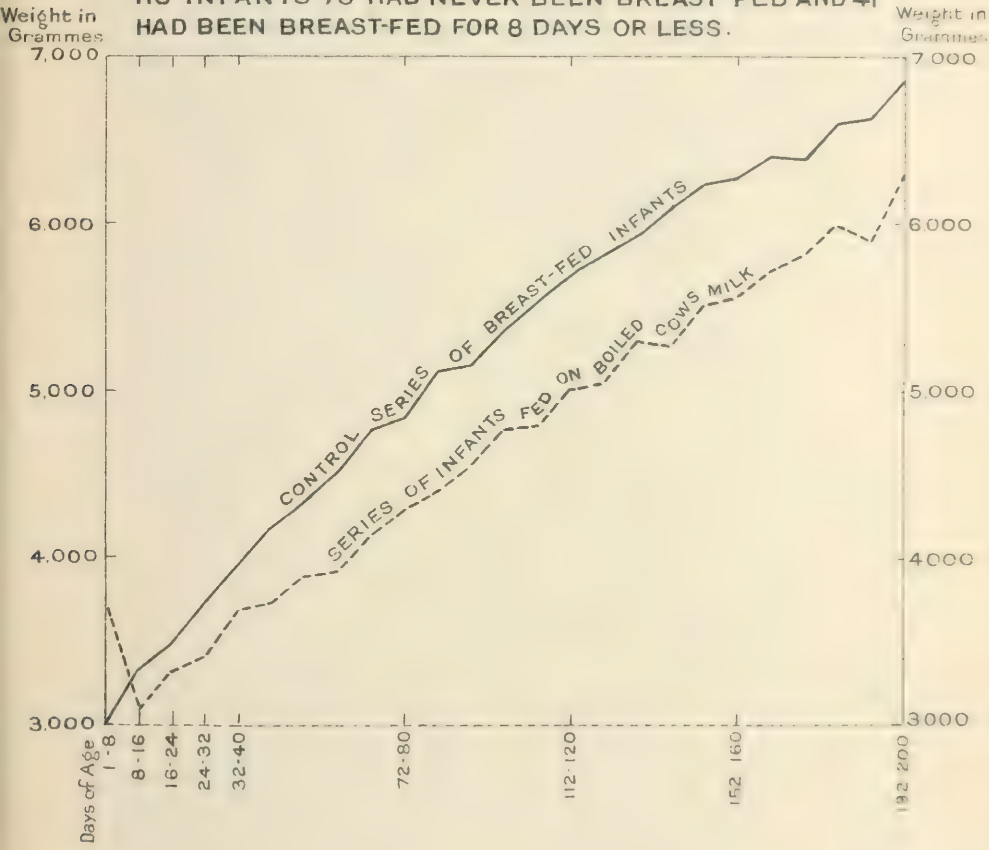

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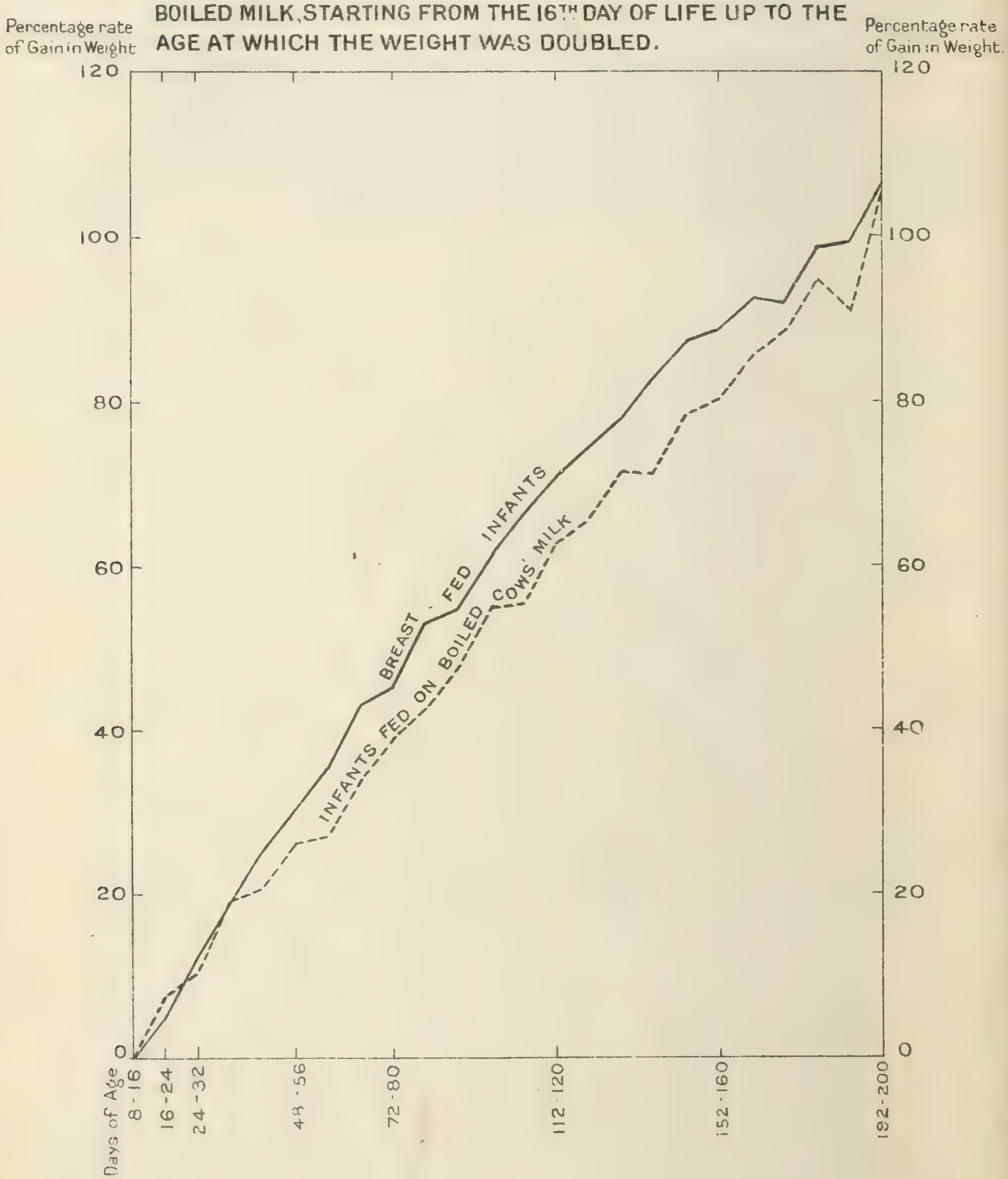
DIAGRAM V.

SHOWING THE PERCENTAGE RATE OF GROWTH OF 130 INFANTS EXCLUSIVELY BREAST-FED & OF 119 INFANTS EXCLUSIVELY FED ON BOILED MILK, STARTING FROM THE 16TH DAY OF LIFE UP TO THE AGE AT WHICH THE WEIGHT WAS DOUBLED.



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Report to the Local Government Board upon the "Biological Properties" of Milk, both of the Human Species, and of Cows, considered in Special Relation to the Feeding of Infants. By Janet E. Lane-Claypon, M.D., D.Sc. (Lond.).

ARTHUR NEWSHOLME,
Medical Officer,
4th January, 1913.

Part I. Introduction.
Part II. The Ferments of Milk.
  Division A. The Presence and Origin of the Ferments.
  Division B. Summary of Division A.
  Division C. Their Probable Value to the Infant.
Part III. The so-called "Protective Substances" of Milk.
  Division A. Their Presence.
  Division B. On their Absorption.
Part IV. General Summary and Conclusions.
Addendum.
Bibliography.[*]
Index.

PART I.

INTRODUCTORY.

The great development which has occurred during the last quarter of a century in the various branches of Public Health, has been manifested to a high degree in the subject of Infant Feeding in relation to Infantile Mortality. The practice of boiling milk was introduced as a result of the discovery that the unboiled milk of a diseased animal when taken as food, could transmit disease.

In 1898, in connection with the fight against tuberculosis in Denmark, regulations were issued for the compulsory pasteurisation of milk, with a view to limiting the spread of this disease. In order to ensure that these regulations were carried out, it was essential to be able to test the milk, so as to detect any infringement of the regulations by the dairyman. For this purpose the property possessed by raw milk of giving colour reactions with certain substances, was utilised, this property being lost when the

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[*] The small numbers in brackets throughout this Report refer to the Bibliography on pp. 81–94.

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milk had been heated to a definite temperature. These colour reactions are due to the presence of ferments, which are destroyed by different degrees of heat, and they will be dealt with fully in the next section. The mechanism of these reactions has been the subject of repeated research and of considerable controversy. The literature is voluminous, and much of it of but little value. The variations in the reactions as to colour with different reagents, the age of the milk, its cleanliness or dirtiness, and the precise temperature at which the reactions cease to be given, have all formed the subject of work, one investigator often rejecting the work of previous investigators, the general result being a bewildering mass of disputed details.

The realisation, towards the latter part of the 19th century, of the high rate of infant mortality which prevailed in most of the countries in Europe, led to a great increase in clinical observation on the feeding of infants.

Experience showed that those babies which received boiled cows' milk did not as a whole thrive so well as those which received raw mothers' milk, and it was first suggested by Escherich(61) in 1900 that the cause of this difference might lie in the destruction of certain substances in the milk by the process of boiling. At that date very little was known about these substances, whatever they might be, and they were termed by Escherich "Stoffwechselfermente" or literally "Metabolism-ferments." The idea, once started, opened up a vast field for investigation, and numerous observers were not slow to enter it.

It was found that milk contains a number of ferments, and it was believed that the destruction of these by boiling might be responsible for the different results obtained with breast- and artificial-feeding respectively. Ehrlich(59) and others had shown that passive immunity could be produced by suckling when the mother was immune: this led to the investigation of the presence in milk of precipitins, agglutinins, and other so-called "Protective substances" in milk.

These bodies, together with the ferments, form the group of so-called "Biological substances" in contradistinction to the more tangible chemical substances (sugar, protein, fats, salts and water) which constitute the directly nutritive part of milk.

One source of error permeates the greater part of the work upon the ferments of milk, namely, the simultaneous presence of bacteria, which produce ferments as a result of their activity. Such ferments differ in no appreciable point from those present in the organs of mammals, and might therefore be expected to occur, in a fluid, such as milk, derived from a gland.

The aim of this report is to investigate the presence of the biological substances; to discover how far they are due to bacterial contamination, and how far they are present in milk as such, apart from the contained bacteria; further, to endeavour to determine whether such substances can be considered to be of value to the infant, in aiding, or bringing about the digestion of the food-material of milk itself, or in producing immunity from the disease.

These biological substances will be dealt with in two groups-

1. The Ferments, and
2. The so-called "Protective Substances."

In dealing with the first group, for the sake of simplifying some of the difficulties, the literature and the original work carried out for the purpose of this report, will be taken together. Some of the work had already been carried out in conjunction with Dr. Harden before the commencement of this report, but for various reasons, the publication has been delayed, and the work, although carried out in 1911 has only recently appeared. The amount of original work required has been still further reduced owing to the recent appearance of several reliable papers (notably Rullmann's(149)), since the work for this report was commenced.

PART II.

ON THE PRESENCE OF FERMENTS IN MILK.

The history of the discovery of the ferments found to be present in milk, is intimately associated with the evolution of our present knowledge of the theory of ferment action.

The mechanism of the activity of ferment has been worked at both in plants and animals. It has been shown that all living cells contain ferments, and that although some of these ferments are highly specialised, and found only in one particular class of either animal or vegetable kingdom, yet there are certain main types of ferments which are found almost everywhere in nature.

At first, as the different reactions became known it was supposed that many of them were different manifestations of the same ferment action; but investigation of their thermal inactivation point, and of their distribution in nature showed that they were due to different ferments. This error has led to much confusion especially in the earlier literature, a confusion which has been further increased by the variety of terms which have been applied to the several reactions, owing very largely to the mechanism of their activity not having been understood.

At the present time, however, the nomenclature of the various reactions is fairly well established, and there is no apparent advantage in using any other terms than those now most generally recognised. Throughout this report, therefore, in dealing with the various ferments, only one name will be used for each ferment-action, even although the authors quoted may, some of them, have used another name.

The ferments which have been described as occurring in milk can be placed, roughly, under the following headings:—

1. Oxidising ferments.
2. Catalase.
3. Reducing ferments.
4. Proteolytic ferments.
5. Lipolytic ferments.
6. Lactase and glycolytic ferments.
7. Amylase.

In the following pages each ferment or group of ferments will be considered separately, and the progress of our knowledge concerning them will be traced down to the present day. Much of the literature is at the present time valueless, for it has been shown to be based upon investigations conducted without due consideration of the effects of bacterial contamination. Some authors have recently been able to eliminate this difficulty, and adopting similar methods, I have been able either to confirm the work of previous observers, or to test for the presence or absence of ferments not hitherto investigated upon these lines. At the same time these earlier papers, now chiefly of historical interest, have undoubtedly been stepping-stones to a clearer knowledge of the mode of action of these bodies.

On the clinical side it must, I think, be admitted that undue importance has frequently been attached to the ferments in milk, without sufficient consideration or investigation of their presence or possible origin.

It will be necessary to consider the ferments which have been found in the milk of different species; the most important milks for the present purpose are human and cows' milk, both of which will be dealt with fully, and the other milks briefly, and only in so far as their ferment content is known.

I.—The Oxidases.

The literature dealing with the presence in milk of oxidising ferments is very large, owing to several considerations. In the first place there has been considerable difference of opinion among investigators both as to the number of bodies which were to be classed among the oxidising ferments in milk, and further as to the specificity of these bodies. Secondly, the presence or absence of the reaction for one of those bodies has been extensively used as a test for the heating of milk, and since this was used in connection with legislative measures in Denmark, it was essential that the test should be reliable. There has again been much disagreement as to the most reliable form in which to apply the test for this body, and as to the mechanism of its action.

In order to simplify the somewhat complicated literature it was considered advisable to divide the question of the oxidising ferments into several sections, and to consider:—

1. The number of oxidising ferments stated to be present,
2. The tests employed for their identification,
3. The effect of heat upon the reaction used,
4. The mechanism of the reaction,
5. The presence of the peroxidase reaction in cows' milk, and
6. The presence of the reaction in human and other milks.

The literature dealing with the first four points will not be taken fully, only such work as is necessary for the essential facts being considered.

(1.)—*On the number of oxidising ferments stated to be present.*

Klebs (1868)(87) seems to have been the first observer to discover the presence of an oxidising body in cows' milk. He showed that fresh milk coloured guaiacum tincture blue, that the same reaction could be obtained with both blood-protein and pus, and that in milk this reaction was connected with the protein. In the next year, Schaer(156) (1869) pointed out that blueing of guaiac tincture could be obtained with certain salts, and that this reaction was one of oxidation, and also occurred if hydrogen peroxide were added. The process he believed to be one rather of ozonisation, and he showed that this body had the power of decomposing potassium-iodide-starch solution, with the liberation of iodine.

In 1881 Arnold(3) showed that this reaction was destroyed in milk by heating to 80° C.

Babcock and Russell(8, 9) (1889) found that the ferment solution prepared by them gave the reaction with guaicum, and they believed that it was another manifestation of the proteolytic activity of their solution (*cp.* p. 42), as was also the catalase reaction given by the same solution (*cp.* p. 15).

The separate identity of catalase and of the oxidising ferment was not recognised until some time later, and much of the literature even down to recent years shows confusion of thought and work between the two bodies. Others have, however, clearly recognised the difference between them, and among earlier authors Raudnitz(133) (1898) stated his belief that the two reactions were due to two separate bodies.

Loew(105) (1901) showed that solutions could be prepared from plants and other tissues which did not give both reactions, but only one of them, and that the reactions were therefore almost certainly due to different ferments. Further Neumann-Wender (120, 121, 122) (1903) showed that the thermal inactivation-point for the two reactions when present in the same solution, namely, milk, or in Babcock's ferment solution, were quite distinct. The separate identity and specific actions of the two were therefore established. It will be considered from this point onwards that the catalase and the oxidising reactions are separate, and attention will now be concentrated upon the latter.

Kowalewsky(96) (1890) carrying on the work of earlier observers upon the action of milk towards guaiac tincture, showed that the reaction was most reliable when old guaiac tincture, which had stood in the light, was used, and Bourquelot([illegible]) (1897) showed that unless old tincture was used it was necessary to add hydrogen peroxide in order to obtain the reaction. Bourquelot believed that guaiacum must contain an aut-oxidisable substance, which became oxidised on standing. Following on these and other similar investigations it became recognised that the blueing of milk with guaiac tincture alone, was unreliable. This was thought to be explicable by the presence in milk of two oxidising ferments.

one of which was capable of acting with guaiac tincture alone, and was only irregularly present, and the other only capable of acting with the assistance of hydrogen peroxide. For these reasons the first of these bodies gradually came to be looked upon as a "direct" oxidising ferment or "direct oxidase" and the other as an "indirect oxidase" or "peroxidase."

Bach and Chodat([15, 16, 17, 18]) (1903 and after) in a series of papers have suggested that there are two ferments, the one which they call "oxygenase" and which from the context appears to be a peroxide, and not a ferment, and "peroxidase" which activates the peroxide or the "oxygenase," and gives rise to the blueing of the oxidisable substance. This suggestion appears to have been very largely accepted by subsequent writers.

Recently (1909) Moore and Whitley([117]) have shown that the oxygenase of Bach and Chodat is really a peroxide, and that when an apparent "direct oxidase" is found, it is due to the presence in the extract of a peroxide, so that the addition of hydrogen peroxide is unnecessary; when however the indirect oxidase action occurs, then there is no peroxide present, but it must be supplied by the addition of hydrogen peroxide.

Without considering the details of this most interesting work it will for the present be considered that there is only one ferment concerned in the production of the oxidase reactions, and the term "peroxidase" will be retained for this body. In dealing with the literature the question of the presence of an oxidase will be ignored, and the peroxidase reaction alone considered, whatever term may have been applied to the reaction by the author. The second point may now be considered, namely, the tests which have been employed for the identification of the presence of this body.

(2.)—*The Peroxidase Tests.*

In the years 1897 and 1898 the test for peroxidase in milk suddenly sprang into great importance. In France in the year 1897 an outbreak of foot-and-mouth disease occurred which was shown to be transmitted by the milk. An order therefore was issued for the boiling of all milk before it was sold, and in order to control the milk-purveyors it was necessary to have some reliable test which could be applied easily and rapidly to test the previous heating of the milk sold. Bourquelot([43]) in working upon the test with this object used guaiacum tincture and found that it was necessary to add hydrogen peroxide if the reaction was to be reliable.

In 1898, with the object of controlling bovine tuberculosis, it was enacted in Denmark that all milk must be pasteurised before it was sold. Storch was appointed to work out a reliable test which could be used to differentiate raw from heated milk. Storch([183]) tried several reagents, and finally decided in favour of para-phenylene-di-amine with the addition of hydrogen peroxide. This test is known as Storch's test, and when carried out in fresh milk gives deep blue-grey colour almost instantly, on the addition of the reagent. In boiled milk, however, the reaction is negative or delayed, and Storch believed that this test was quite reliable for differentiating between milk which has been heated to 80° C. and over, and milk which had not been heated to so high a temperature.

It was inevitable that considerable discussion should arise about so important a reaction dealing with public health matters, and the literature upon the details of the peroxidase reaction is voluminous. Many observers still preferred the guaiac reaction even after Storch's test was published, and they endeavoured to find some means which would render the reaction reliable. Some, like Kowalewsky and Bourquelot, believed that the use of old tincture was the best plan; others preferred to use guaiacum bark and others the guaiacum resin: Seigfeld (170, 171) advocated the use of an acetone solution of guaiacum, which he believed to be always reliable.

Much discussion was aroused by the introduction of Ursol D. as a reagent (*cp.* Arnold and Menzel(4), Glage(69) and v. Itallie(82), Weber(203), Wirthle(206), Zink(209), Chlopin(65) and Utz(189)) in the place of para-phenylene-di-amine; many other substances which strike a colour on being oxidised have been suggested and to some extent used.

As a whole it seems that the guaiac reaction and Storch's reaction have been the most widely used, Kastle and Porch(86) (1908) found that Storch's reaction could be sensitised by the addition of a one per cent. solution of trikresol, and Rothenfusser (1908 and 1910) found para-phenylene-di-amine-hydrochloride more reliable than the base itself. Rothenfusser(144, 145) further suggested a mixture of guaiacol and para-phenylene-di-amine as a reagent. This mixture strikes a brillant violet with raw milk, and is known as Rothenfusser's reagent. The serum obtained after treating milk with lead acetate is recommended by the author to be used for the test. Before dealing with the cause of this reaction the effect of temperature upon the peroxidase reaction in milk must be considered.

(3.)—*The Effect of Heat upon the Peroxidase Reaction.*

In using this reaction for the purpose of detecting the previous heating of milk it is evidently necessary to know at what temperature this reaction ceases to be given. The numerous observations upon this point are by no means concordant. It is not possible to deal in detail with the various results obtained, but the accompanying table, compiled in great measure from the tables given by Waentig(200) (1907) and van Eck(48) (1911), will show the most essential points. It shows the great difficulty of deciding upon the precise temperature at which the reaction ceases to be given, and therefore the exact temperature to which the milk may be considered to have been heated.

Table showing the Inactivation-point for Peroxidase, after different Authors.

Author.	Reaction persists at	Is destroyed by temperature of
Dupouy(56)	... ...	80° C.
Storch(183)	75° C. for 2 minutes ...	79–80° C.
Leffmann(100) ...	76·5° C. (still active) ...	82° C.
Tjaden, Koske, Hartel(185)	Below 80° C	Over 90° C. Depends upon time.
Rullmann(146, 147) ...	½ hour at 70° C. (weak after 5 minutes).	75° C. for 10 minutes or 69–70° C. for 1 hour.
Schweitzer(162) ...	About 1 hour at 65° C. ...	
Utz(189, 190)	71° C. for 1¼ hours ...	
Neumann-Wender(120)	... ...	83° C.
Seligmann(168) ...	... ...	72° C. for 15 minutes.
	... ...	75° C. for 5 minutes.
	... ...	76° C. for 1 minute.
Butterberg(49) ...	... ...	70° C. for 30 minutes.
Koning(88)	86° C. if heated quickly ...	73–74° C. if heated slowly.
Kastel and Porch(86)	... ...	70° C. for 1 hour.
	... ...	75° C. for 20 minutes.
Giffhorn(67)	... ...	72° C. for 30 minutes.
Van Eck(58)	Depends upon time of heating.	

Van Eck (1911) arranged a series of test tubes containing varying amounts of raw sterilised milk. (The sterilised milk was heated to 100° C. for ½ hour.) The series ranged from 9·9 cc. sterilised milk and ·1 cc. raw, to 8 cc. raw and 2 cc. sterilised. After adding equal amounts of the reagent (Storch's test was used) these tubes formed a scale of colour which was used for each experiment as a standard colour scale, the colour depending upon the amount of fresh milk, that is upon the amount of ferment present in each tube.

He then took some of the same milk and heated it gradually in a water-bath fitted with a stirrer, and, taking samples at different temperatures and intervals, he compared them with the standard colour scale for that experiment.

Using the values thus obtained he found that the reaction followed the law for monomolecular reactions, and that the equation $K = \frac{1}{T} \log_e \frac{a}{a - x}$ (where K = a constant, and a = concentration at time 0, x = concentration at time T), was true, and that hence the reaction was monomolecular in type. The effect evidently depends upon the length of time over which the heat is maintained, and van Eck points out that it is impossible with any one test to discover both the temperature and the length of time of heating.

There is no means of testing the amount of peroxidase present in milk; van Eck's work deals with the disappearance of such amounts of peroxidase as are present in the particular sample of milk considered, and not with absolute quantities. Further, the test for peroxidase can readily be restored to heated milk by the addition of a small amount of raw milk, if the presence of the reaction is required.

(4.)—*The Mechanism of the Peroxidase Reaction.*

The oxidising ferments have always attracted much interest among scientific workers, but, although a large amount of work has been done upon them, it cannot be said that their action is fully understood.

It appears that at any rate one cause for the production of the peroxidase reaction is due to the presence of either iron or manganese, probably in colloidal form, since a metal sol will also give the reaction.

Bertrand(34, 35) (1897) showed that the oxidising ferment which produces the Japanese lacquer acts only when manganese is present, and Sarthou(152) (1900) found that another oxidising ferment obtained from a plant owed its activity to the presence of iron.

Engler and Wohler(60) (1904) suggested a theory whereby the action of the ferments in milk are brought into line with the action of a metallic sol. This suggestion was carried further by Bordas and Touplain(40, 41) (1909). They showed that the ferment reactions of milk are given by colloidal solutions of metals, and that these same reactions can also be given by solutions of lactate and oxalate of iron. They also showed that milk, if heated to 85° C. and then pulverised by being projected in a fine jet under high pressure on to an agate plate, would again give the reactions for peroxidase and catalase, and they believed this to be due to the physical condition of the milk. They found that milk could also be re-activated by pumice. J. Meyer(115) (1910) confirmed the findings of previous observers and showed that boiled milk could be re-activated by the addition of pumice, kaolin, or better still, platinsol.

Moore and Whitley(117) found iron and manganese constantly present in the peroxidase solution made by them from potato juice; the only observer who failed to find either one or other metal in such a solution was (as far as I can ascertain) Bach(18) (1910), who, however, gives no details of the method used nor of the degree of accuracy of his tests.

Van der Haar(74) (1910) prepared an active peroxidase solution from the leaves of Hedera Helix and from potato juice. The solution always contained manganese in varying proportions (from ·00023 per cent. to ·03 per cent.), the amount not varying directly with the strength of the ferment reaction.

Grimmer(71) (1911 and 1912) prepared a peroxidase solution from milk. The ash of this solution was found to always contain iron and frequently manganese, this last in very small amounts.

Sarthou(154) (1911) showed that the peroxidase reaction may be brought about by infinitesimally small amounts of colloidal iron or manganese, the action of the latter being assisted by the presence of iron: a solution containing ·0002 per cent. of iron is sufficiently strong to bring about the reaction.

It has also been shown by several observers that the guaiac reaction can be given by other salts, especially chlorides. Thus, Alsberg(2) (1908), working with the blood of sea-water animals, showed that sodium and potassium chlorides acted as sensitisers for the peroxidase reaction, and that the intensity of the action varied with the concentration of the salt. He believed the guaiac test to be entirely unreliable. Sarthou(151) and Sartory(155) (1911) both pointed out the same facts. Sartory worked with the water of Breuil and showed that this mineral water even after being bottled for two years will still give the peroxidase reaction with guaiac and hydrogen peroxide, and has catalytic properties.

There is no difficulty in supposing that the salts of iron and possibly manganese are concerned in the peroxidase reaction in milk. It has been shown that Grimmer found these last two metals in the solution made by him from milk, and iron is well known to be constantly present in both human and cows' milk, but I am unaware of any evidence as to the condition of the metal, whether colloidal or not. The most recent work upon the iron in milk is that of Langstein(99) (1911), in whose paper further references can be found. Several authors have shown that the peroxidase reaction depends upon the alkalinity of the milk. Thus Kooper(91) (1910) and Hesse and Kooper(93) (1911) showed that as the acidity of milk increased the peroxidase reaction became weaker. They worked with Rothenfusser's reagent and showed that the alkalinity of the milk decreases on boiling and the reaction disappears. If, however, a little alkali is now added the reaction at once returns. This is, however, not the case if the alkali is added before boiling; in this case the alkali appears to be bound, for the total alkalinity is decreased, and the reaction is not given.

Grimmer(71) (1911 and 1912) did not entirely agree with Hesse and Kooper, for he found that if the milk is heated above 100° C. the addition of ammonia did not restore the reaction. He prepared a solution by dissolving the precipitate obtained by the complete saturation of whey with ammonium sulphate and found that this gave a strong peroxidase reaction. He concludes that the ferment must either be of the nature of lact-albumin or that it must be absorbed by the protein. The reaction was abolished if the alkalinity of the solution was raised to that of a $\frac{N}{40}$ soda solution. The ferment action was also entirely destroyed by digestion with either pepsin or trypsin.

One more point remains for consideration which has been already mentioned on p. 6, namely, that of the relation of the peroxidase to a peroxide. Neumann-Wender(120) (1903) found that active guaiac tincture, that is, one giving the "direct" oxidase reaction, was inactivate by boiling, and he believed that the active tincture contained a peroxide. That this is the case was definitely shown by the work of both Arnost(5) (1905) and Waentig(201) (1907). Guaiacum contains a substance which becomes oxidised on exposure to the air, and forms a peroxide. If this substance is formed in sufficient quantity the tincture is "active," and the peroxidase reaction is given without the addition of hydrogen peroxide. When guaiacum is digested with acetone, as was done by Siegfeld in making his active solution, this substance is formed.

The work of Bach and Chodat has been mentioned already on p. 6. From their work and from that of Moore and Whitley(117)

(1909) it appears that a peroxide is present in many of the plant juices. Moore and Whitley worked with vegetable extracts and also with milk. An active plant juice which requires no addition of peroxide when quite fresh becomes inactive on standing or warming to 50-60° C., the reaction being then only given when hydrogen peroxide is added. They prepared a solution of peroxidase from the juice of potatoes and found that the solution was colloidal in character, reduced Fehling solution, did not give the protein tests, and was completely destroyed by an acid or by alkali in more than the merest trace.

The peroxidase reaction seems, therefore, to be dependent for its production upon the reaction of the medium, and probably upon the presence of a metal in colloidal condition. The reaction can also be brought about by certain salts.

(5.)—*On the Presence of the Peroxidase Reaction in Cows' Milk.*

It has already been stated that no observer has failed to find the reaction for peroxidase present in cows' milk, and it only remains therefore to consider the possibility of the reaction being due to bacteria, and the portion of the milk to which the body giving this reaction is attached.

As regards the first point it has been shown by numerous observers, apart from the evidence already given in the previous section, that the reaction disappears as the alkalinity of the milk becomes lessened; this is somewhat against its bacterial origin although not entirely, since it is well known that the bacteria themselves die as a result of the increasing acidity of the milk brought about by their own activity. The only observer who appears to have obtained a positive result with bacteria is Bellei(31) (1904), who found the peroxidase reaction positive in milk previously boiled and then inoculated with B. Pyocyaneus, though the reaction was slower than in fresh milk. Much stress cannot be laid upon this observation, which has not been confirmed by any subsequent observer.

Jensen(83) (1906) tried to detect the peroxidase reaction in bacterial cultures of the commoner milk organisms, including some of the lactic-acid-forming varieties, the results being in all cases negative. Oidium Lactis and Penicillium Glaucum showed a blue colour inside the hyphae, but not outside, thus showing that the ferment was present inside the cell of the plant. He thinks, therefore, that the peroxidase reaction in milk is not due to bacteria.

Barthel(23) (1907) also showed that milk rendered sterile by chloroform and toluol gives a positive reaction for peroxidase.

Rullmann(149) (1911) working with initially sterile cows' milk found the peroxidase reaction always positive, and Harden and Lane-Claypon(74) (1912) working with the same class of milk obtained identical results.

It appears therefore that the peroxidase reaction is not due to bacterial contamination.

As regards the part of the milk concerned with the production of this reaction, the evidence already given shows conclusively that it is concerned either with the lactalbumin or with some similar body.

Klebs(87) (1868) as already mentioned (*see* p. 5), believed it to be due to the caseinogen, whilst Babcock and Russell(9) (1898) found that the slime obtained on centrifuging milk gave a very strong reaction.

Kowalewsky(96) (1890) believed that the reaction was connected with the lactalbumin, and Raudnitz(133) (1898) found that the active substance was precipitated with different fractions according to the precipitant used. Later (1903) he considered that it came down with the caseinogen, but was not attached to it.

Barthel(21) (1899) thought that the reaction was dependent upon the caseinogen as also did Bordas and Touplain(40, 41), who considered that the whole reaction was a function of the caseinogenate of calcium and not of a ferment at all. They found also that both the cream and sediment of boiled milk gave the reaction. Monvoisin(112) (1908) found the reaction positive in the filtrate obtained after saturation with magnesium sulphate, that is after the caseinogen had been removed; hence it is not dependent upon the globulin fraction.

Meyer(110) (1910) criticising Bordas and Touplain's work showed that caseinogen alone, whether prepared by 24 hours' incubation, by the addition of tri-chlor-acetic acid, or by rennet, from either raw or boiled milk did not give the peroxidase reaction. He obtained a positive reaction with the cream from centrifuged milk as well as with the fluid, and a weak effect with the sediment.

Kooper(91) (1910) found that the peroxidase remained behind in the fluid on centrifuging, and did not go up with the cream; and Sames(150) in the same year showed that it was probably attached to the albumin molecule and that the reaction was strongest in the first milk and often absent in the strippings.

If to these results the work of Grimmer recorded in the previous section (*see* p. 10) be added it seems fairly evident that at any rate the major part of the substance bringing about the peroxidase reaction must be associated with the albumen fraction. Weber(204) (1910) found " oxidase " present in colostrum, except sometimes on the first day after birth. Giffhorn(67) (1911) found the reaction in cows' milk much stronger in cases of mastitis, than in normal milk.

Grimmer(70) (1910) in his work upon the mammary glands of various animals found that the glycerine extract, made without injury to the cells, was invariably negative to the peroxidase test, except in one case in which there was mastitis. If, however, the glands were ground up with quartz, then the result was in all cases positive, whether the gland was lactating or not, even in the case of the mare and sow, whose milk did not give the peroxidase reaction (*see* p. 14).

The results of sections (5) and (6) will be summarised together,

(6.)—*On the presence of the Peroxidase Reaction in Human Milk.*

Raudnitz (1898) failed to obtain the peroxidase reaction with human milk using guaiacum and hydrogen peroxide; and showed that this was not due to any inhibitory body, since when human milk was added to cows' milk the reaction with the cows' milk took place just as easily. He found, however, that human colostrum was active, and that the active substance was precipitated with the globulin fraction. It may be pointed that Raudnitz found the same for cows' milk, in respect of this last point.

Moro(113) (1902) using the oxidation of salicylic aldehyde as a measure of the oxidising power, obtained negative results with human milk.

Spolverini(180, 181) (1902 and 1904) found a weak reaction with human milk, and showed that it became stronger if the milk assumed the character of colostrum. He believed that the ferment was attached to the leucocytes.

Nordmann(127) (1902) published a case of a child who was fed upon the breast of a mother who was suffering from mastitis. The infant did not thrive, and on testing the milk it was found to be negative to Storch's reagent. Three samples of milk taken from other women gave a positive reaction, and Nordmann apparently attributed the infant's lack of progress to the absence of peroxidase. This paper produced a reply from Thiemich([illegible]) (1903), who showed that the presence of peroxidase is altogether uncertain. He examined the milk of a large number of women attending the Breslauer Klinik, and found that whilst it was usually present it was very inconstant.

Marfan and Gillet(109) (1902) found that the reaction is present in colostrum, but disappears as the gland gets into full work; if, however, the gland is allowed to become less active, then the peroxidase again appears, and with it the leucocytes; the peroxidase reaction may, however, appear rather earlier than the leucocytes. They further found that if milk giving the peroxidase reaction be examined under the microscope, on the addition of guaiacum and hydrogen peroxide, the area around the nucleus of the polymorpho-nuclear leucocytes became blue. The fluid around also showed a faint bluish tinge, so that, apparently, the substance can pass out.

Gillet(83) carrying this work further, showed that the presence of peroxidase is due to leucocytes, and that it is found both in the fluid and in the sediment of centrifuged milk.

He also examined the milk from a large number of women, and found, as Thiemich did, that the reaction was extremely uncertain; it varied in the milk from the two breasts.

Friedjung and Hecht([illegible]) (1903) working on the milk from a large number of women, found that the reaction was negative in 114 out of 174 samples of milk examined, and varied greatly in intensity. As a whole the reaction was more marked in colostral milk, but it was often absent even here.

Jolles(84) (1904) was so entirely unable to detect the presence of peroxidase in human milk that he suggested that the test should be used as a means of distinguishing cows' milk from human milk.

Kastle and Porch(⁸⁶) (1908) found the results of the peroxidase reaction very uncertain with human milk, even with the addition of their sensitiser trikresol; the colostral stage appeared to have, as a whole, greater activity than the later milk.

The peroxidase reaction has been found positive in goats' milk, by Spolverini(¹⁷⁹) (1902), Luzzati and Biolchini(³⁸) (1902), Raudnitz(¹³³) (1898), Grimmer(⁷⁰) (1910), Harden and Lane-Claypon(⁷⁵) (1912), and by Weber(²⁰⁴) (1910), who worked with colostrum only.

It was positive in sheeps' milk. (*Cp.* Raudnitz, Grimmer, and Weber.)

Raudnitz (1898) found the reaction negative in the ordinary milk of the horse, dog, ass, and rabbit, but positive in the colostrum of these animals.

Spolverini (1902) found it weakly positive in the milk of dogs and asses, and Grimmer found it negative in the milk of the horse and pig.

Reviewing the work described in sections 5 and 6, it appears that peroxidase is constantly present in the milk of cows, and only inconstantly present in human milk. There is no reason to suppose that it is bacterial in origin.

Comparing the work of Weber, Giffhorn, Grimmer, Spolverini, and Marfan and Gillet, it appears that peroxidase is derived from cellular elements, and at any rate in great part, from the leucocytes.

2.—Catalase.

The name catalase is now very widely, if not universally, applied to the ferment, generally found in both animal and vegetable tissues, having the property of splitting hydrogen peroxide into water and oxygen, with the formation of molecular or inactive oxygen, according to the equation—

$$2H_2O_2 = 2H_2O + O_2.$$

The name of this ferment has been the subject of considerable discussion, and it has not infrequently been called superoxydase; in this report, however, the more generally accepted nomenclature will be followed throughout, and the ferment will be termed catalase, even in dealing with papers by authors who prefer the other term.

The literature dealing with the presence of this body in the milk of various animals is extremely voluminous, and much of it does not present sufficient interest for it to be necessary to deal with it in detail. Many of the papers are concerned with the attempt to find a relationship between the catalase content and the purity or otherwise of the milk; this aspect will be referred to somewhat briefly, as it does not come strictly within the scope of the present investigation.

On the Presence of Catalase in Cows' Milk.

The existence of this ferment was known for many years before its presence in milk was detected.

In 1882 (²⁷, ²⁸, ²⁹), Bécamp and Bert and Regnard(³³), showed that this property was common to extracts of practically all the tissues they examined. Bécamp showed that fibrin was very rich in this substance, and that it could split many times its own volume of hydrogen peroxide.

Babcock(⁷) (1889) discovered that milk decomposed hydrogen peroxide in varying quantities and, apparently owing to the work of Bécamp, concluded that fibrin must be present in milk, although he was quite unable to demonstrate its presence. Babcock also found that the strippings were the most active parts of the milk in this reaction, but he did not think that there was any definite relationship between the intensity of the reaction and the fat content, since the action persisted even after the cream had been removed. Colostrum was from 10 to 15 times as active as ordinary milk.

Babcock pointed out that in estimating the amount of gas evolved by the action of the ferment, it was necessary to shake constantly in order to obtain accurate results.

Later, Babcock working with Russell(⁸, ⁹) on the ripening of cheese, prepared a ferment solution from milk which had proteolytic activities, and also had the power of spliting hydrogen peroxide. They concluded that this was another aspect of the activity of the proteolytic ferment; later again, they found that this solution also gave the peroxidase reaction, and considered that this last reaction could be taken as a measure of the catalytic activity of the solution.

Raudnitz(¹³³) (1898) found catalase present in cows' milk, and considered that it must be different from peroxidase because it was present in human milk (*cp.* p. 13). Also he showed that peroxidase and catalase had different reactions towards precipitating agents.

Lépinois(¹⁰³) (1899) came to the same conclusions as Raudnitz, namely, that the different reactions are due to two ferments, because in solutions of tissue extracts which gave both the catalase and the peroxidase reactions, (*a*) heating to 70° C., destroyed the power of the solution to split hydrogen peroxide, but did not affect the oxidising power, and (*b*) in fresh extracts if alcohol be used as a precipitant it is possible to obtain a precipitate which has catalysing but not oxidising powers.

Storch(¹⁸³) (1899), who apparently did not consider that the reactions were due to separate bodies, was able to prepare a solution of lactalbumin, which had been dried at 40° C. and showed catalytic but not oxidising power.

Barthel(²¹) (1899) considered that the catalytic action described by Babcock and Russell was due to the leucocytes, which were not, however, the only constituents of the blood to possess this property.

In 1901 Loew(¹⁰⁵) published his well-known paper on catalase, which, although it does not deal directly with the catalase of milk, contains many points which are of sufficient importance for it to be necessary to deal briefly with his work. He suggested the term catalase for this ferment, and subsequently upheld this term in preference to "superoxydase."

Loew showed that the power of splitting hydrogen peroxide must be distinct from that of oxidising, since extracts could be prepared which showed the presence of catalase and not of peroxidase and *vice versa*.

Dealing with the occurrence of catalase, he says:—

> "Numerous tests have established beyond a doubt that catalase is of general occurrence in the vegetable kingdom. No living plant or vegetable organ tested was found free from it." And also, "In the animal kingdom catalase is also of universal occurrence. The aqueous extracts of spleen, pancreas, liver, kidney, brain, muscle and blood serum show the power of catalysing hydrogen peroxide."

Loew also found that bacteria were capable of producing catalase, notably B. Pyocyaneus. In estimating the amount of oxygen evolved by catalase, he emphasises the importance of continuous shaking.

In a further paper, Loew deals with the probable importance of catalase in the tissue extracts. He believes its presence to be essential for the removal of hydrogen peroxide, which appears to be present in the living cell, and yet to be harmful to it. In this view he differed from Bach and Chodat, who considered that hydrogen peroxide was not harmful to living tissue.

Chick(52) (1901) worked upon the presence of catalase in milk, in the course of some work undertaken in order to test the value of hydrogen peroxide as a preservative for milk. This method of sterilising milk had recently been suggested by Buddé, and the process is, therefore, known as Buddisation. She showed that milk when raw had the power of splitting hydrogen peroxide, but that this power was lost upon the addition of fairly large quantities of the reagent. when sterilisation of the milk was obtained. If, however, milk which had been sterilised by boiling was inoculated with a little raw milk, the power of splitting hydrogen peroxide, which had been destroyed by the boiling, gradually returned. Both these facts pointed to the production of catalase by bacteria, but it did not at all follow that the catalase was produced by bacteria alone.

Later (1903), Renard(140) found that the catalytic power of milk was inhibited by the addition of 3 per cent. H_2O_2, and that milk which had been heated to 75° C. could be very well preserved by this means.

Some observers about this period had believed that catalase was a reducing ferment, but Bach and Chodat (1903) showed that it was a specific substance whose action was to split hydrogen peroxide, and that only. They prepared substituted peroxides, and found that the ferment did not attack them. They also showed that catalase as such has no reducing power, and that it is quite distinct from oxidising ferments.

This view was further confirmed by Neumann-Wender (1903), who prepared a solution of ferments from milk, after the method of Babcock and Russell, and showed that—

(*a*) the proteolytic power was destroyed by heating to 75° C.
(*b*) ,, catalytic ,, ,, ,, 80° C.
(*c*) ,, oxidising ,, ,, ,, 83° C.

He concludes that the ferment solution of Babcock and Russell was, therefore, composed of a complex of ferments.

From this date it seems to have been fairly well established that catalase is a specific ferment, and it will be dealt with as such in this report.

The consideration of this ferment can most conveniently be divided into several sections:—

1. The source of the catalase in cows' milk.
2. The estimation of catalase.
3. The relation of the catalase content to the condition of the milk.

Section I.—The Source of the Catalase in Cows' Milk.

There appears to be a special affinity between the catalase and the fat content of the milk, since if the milk be centrifuged the major part of the catalase goes up with the cream. The catalase does not, however, appear to be directly attached to the fat globules, since it can be washed away fairly easily. The total amount of catalase present is very variable, as was pointed out by Raudnitz(136) (1903), even when the conditions were kept constant as far as was possible.

Reiss(139) (1905) showed that catalase can be dissolved out from the cream, obtained by centrifuging, by either water or saline, and that the fat globules are not injured. This work was confirmed in the next year by Seligmann, who gave the following figures:—

25 c.c.	skim milk	gave	1·2 c.c.	O_2	with 0·5 c.c.	perhydrol	in 1 hour	at	37° C.
,,	whole milk	,,	4·0 c.c.	,,	,,	,,	,,	,,	,,
,,	cream	,,	7·3 c.c.	,,	,,	,,	,,	,,	,,
	Watery extract from cream	,,	12·0 c c.	,,	,,	,,	,,	,,	,,

Jensen(83) (1906) estimated the oxygen evolved from the different portions of milk, first milk, middle milk, and strippings, and found a well-marked connection between the fat and the catalase content (*see* table on p. 18). He showed that the strippings contained more leucocytes than the rest of the milk, and believed that small quantities of the catalase was derived from the leucocytes, the greater part being bacterial in origin.

Heygendorff and Meurer(78) (1910) also found more catalase in cream than in the rest of the milk. In the same year Meyer(110) showed that catalase is present in the cream, the skim milk, and in the sediment, in the last only in small quantities, unless dirt be present, when it is increased. This was also found by Harden and Lane-Claypon (*see* p. 19), and other observers have obtained similar results.

This does not touch the question of the origin of the catalase, which must now be considered. Chick's work, already quoted, showed that bacteria could produce catalase in milk, and this has been shown by a number of subsequent observers.

Seligmann(164 165, 166) (1905 and 1906) believed that the catalysing and reducing powers of milk were closely connected, and that both were due to bacteria alone. He isolated a strain of cocci from milk, which, on being cultured, gave large quantities of catalase, and considered these to be the source of the catalase in milk.

Koning(88) (1906) found the catalase content very variable, and showed that bacteria were able to produce catalase but not peroxidase, and hence concluded that these were two different bodies.

Jensen(83) (1906) showed that many bacteria will produce catalase, among others Staphylococcus Aureus, Proteus Vulgaris, and P. Zopfii, B. Prodigious, and Oidium Lactis.

He collected milk into sterile tubes with all precautions against bacterial contamination, and estimated the fat and bacterial content, and the oxygen evolved from 10 cc. milk.

The figures given on the following table show that there is a marked connection between the fat and the catalase, but not between the small number of bacteria and the catalase.

—	Fat Content. Per cent.	Bacteria per c.c.	Oxygen from 10 c.c. Milk.
Cow 1:			
First milk	·55	160,000	Trace.
Middle milk	2·70	480	·5 c c.
Strippings	8·30	360	2·0 c.c.
Cow 2:			
First milk	1·5	3,200	·5 c c.
Middle milk	3·4	2,800	1·0 c.c.
Strippings	7·8	360	1·5 c.c.

Sarthou(153) (1909 and 1910) writing against Bordas and Touplain (*see* p. 19) showed that if milk is carefully collected it contains very little catalase immediately after milking, but that on keeping the quantity increases, or, if after heating, the milk is inoculated with a lactic-acid-forming organism.

Kooper(91, 95) (1910 and 1911) showed that the catalase content of milk was greater on the second day after milking than it was on the first, and believed this to be due to the development of the bacteria. If milk was collected with all possible precautions against bacterial contamination, the amount of catalase was much less, but that the content in this milk was greatly increased by the addition of a small piece of solid excreta. He also showed that boiled milk can be re-activated by inoculation with raw milk.

Faitelowitz(61) (1910) agreed with previous observers as to the increases of catalase in milk on keeping. The addition of chloroform or formalin inhibited the increase of catalase, which he believed to be due to bacteria.

There can be little doubt that the greater part of the catalase in milk is bacterial in origin, but it is not yet apparent from the work already given that this is the sole source of the catalase. Barthel(24) and Smidt(173) (1908) working independently both showed that milk which has been rendered sterile by the addition of antiseptics gives the catalase reaction, and believed this to indicate that there was a source of catalase other than bacteria.

This missing link was supplied in 1911 by the work of Rullmann, and has been confirmed by Harden and Lane-Claypon in work undertaken before Rullmann's results were published, but only actually in process of publication at the present time.

Both Rullmann and ourselves obtained the milk by means of the milking-tube; milk thus obtained giving a negative bacterial count.

In 20 samples Rullmann obtained complete sterility, and in others the bacterial count was negligible. He used 20 cc. of milk, and 10 drops of 3 per cent. H_2O_2. Koning's method was employed, and the samples were kept on ice. Summarising his results the sterile samples gave the following O_2 figures:—

After 1 hour—Nil, or only a trace.
After 12-18 hours—0·2-3·8 cc.
After 18-24 hours—0·2-3·6 cc.

In milk which was not entirely germ-free rather more oxygen was evolved after the longer periods of observation than in the case of the sterile samples. Dr. Harden and Lane-Claypon(75) found catalase present in all cases, but the amount of oxygen given off varied within wide limits. The following table gives the summary of the results of the more important experiments:—

Material.	Bacterial Count. Per c.c.	Oxygen Evolved.	Time Allowed.
Exp. 1:			
50 c.c. whole milk	Nil	3·7 c.c.	3 hrs. 50 min.
Exp. 2:			
50 c.c. whole milk	47	6·0 c.c.	3 hrs. 25 min.
Exp. 3:			
50 c.c. whole milk	2·0	12·6	4 hrs.
45 c.c. skim milk	2·0	2·8	4 hrs.
Cream from 45 c.c. milk (the cream was made up to 45 c.c. with sterilised saline).	2·0	4·5	4 hrs.
Exp. 4:			
50 c.c. whole milk	6·0? plates spoiled.	5·9 c.c.	50 mins.
45 c.c. skim milk (no sediment) ...	Ibid.	2·4 c.c.	50 mins.
Cream from 45 c.c.	Ibid.	0·9 c.c.	50 mins.
Exp. 5:			
50 c.c. whole milk	1·0	3·7 c.c.	30 mins.
50 c.c. whole milk (no sediment)...	1·0	2·9 c.c.	30 mins.
Sediment from 50 c.c.	1·0	0·4 c.c.	30 mins.

From this table it will be seen what extremely variable results were obtained with this almost or completely sterile milk.

Much more oxygen was collected by the method finally employed (with the gas apparatus devised by Harden, Young and Thompson) than by any other method used by us. For every 50 cc. milk, 5 cc. of a 1 per cent. solution of H_2O_2 were used, this quantity of milk being much greater than that used by previous observers, so that if a comparison is made between the amounts, this must be allowed for as well as for the fact of the different apparatus.

Before leaving the question of the source of the catalase in cows' milk, the work of Bordas and Touplain must be mentioned; and this rather because of the attention which their work excited, than because of its value for the present report. They apparently looked upon peroxidase and catalase as the same body.

They obtained evidence which led them to believe that the reactions were not destroyed by boiling, and that they were due to the presence of calcium-caseinogenate.

Sarthou (1909) wrote against this view, and pointed out that they gave the reaction for peroxidase and not for catalase.

J. Meyer(110) (1910) also wrote showing that the catalase reaction is positive if caseinogen or casein be prepared by means of trichloracetic acid, heat or acid, and then mixed with water. If, however, the milk is previously boiled, then no catalase reaction is given. The boiled milk can, however, be re-activated by platinsol.

The work of Grimmer(76) (1910) may also be mentioned. This worker extracted the mammary glands of cows' and other animals with glycerine, and found that the catalase content was greater in non-lactating than in lactating glands, but that even in this case it was much greater than in the milk.

Reviewing the work described in this section, it appears that—

1. Catalase is constantly present in cows' milk.
2. That the main source of this catalase in milk is bacterial.
3. That it is found in small quantities in milk collected free from bacterial contamination.

Section 2.—The Estimation of Catalase.

It is quite clear that if the amount of catalase is to be taken as representing the degree of contamination, some method must be adopted of measuring accurately the amount of oxygen evolved by the sample of milk under examination. This, although apparently simple, has proved a difficulty which can hardly be said to have been successfully overcome.

The apparatus must be simple so that it can be easily used in a dairy laboratory; the test must be carried out as quickly as possible, and it must be reliable. A number of pieces of apparatus have been devised for this purpose, of which only the more important will be described.

Thus Koning(88) used a saccharimeter tube, and read off the results obtained.

Burri and Staub(48) introduced a method wherein a small vessel, having a long graduated neck, was used. The neck was filled with a plug of agar-agar, and the milk and hydrogen peroxide inserted through the opposite end. The gas, as it was evolved, pressed up the agar plug, and the amount of oxygen could then be read off.

The Lobeck-Gerber(66) apparatus is the one which is advertised in most catalogues dealing with dairy apparatus. It is a neat, and rather pretty piece of apparatus, and on the surface it seems that it should be accurate.

In some of these methods the essential fact is entirely neglected, namely, that the apparatus must be capable of being frequently shaken during the whole period of investigation.

Kooper(94, 95) (1911) endeavoured to reduce the confusion existing in this direction; he tried to find some constant factor by which the results obtained by one method might be multiplied, so as to make them numerically comparable with those obtained by another method He used four different apparatus, namely, Koning's, Funk's, Henkel's and Gerber's (Lobeck's). Of these Koning's and Henkel's cannot be shaken, Funk's can be shaken, but the gas is mixed with the milk; Lobeck's also can be shaken, and gives the highest reading of the four; this does not, however, necessarily mean that it is the most accurate.

Kooper seems to have carried out this most laborious task with great care, and, working upon a large number of samples, of which he took the average, he believed that he had found factors by which it would be possible to convert the results obtained by one apparatus into terms of another. The figures obtained by him upon which he based his average figure, showed such high differences that it seems doubtful whether the results obtained by this method possess any value. His paper gave rise to a prolonged discussion between himself and Grimmer upon the details of his methods, and the results obtained, into which it is not necessary to enter.

Spindler(^{177}) (1911) used Lobeck's apparatus and found that the amount of gas evolved depended upon the capacity of the apparatus, also upon the temperature, rather more gas being formed at 37° C. than at 25° C. Further the amount of H_2O_2 used had an appreciable effect. As a whole 15 cc. milk and 5 cc. of 1 per cent. H_2O_2 gave the best results, as to absolute quantity; but smaller amounts of milk and H_2O_2 in the same proportions gave relatively larger amounts. Successive additions of 5 cc. of H_2O_2 gave still larger amounts of gas.

Harden and Lane-Claypon(^{75}) (1912) found that neither Koning's nor Lobeck's apparatus was reliable, but apparently satisfactory results were obtained by using the apparatus devised by Harden, Thompson, and Young for the investigation of the gases evolved by growing yeast. This apparatus requires constant shaking, the evolved gases passing into a eudiometer, and being collected over mercury.

Although there are these difficulties connected with the estimation of the oxygen produced by catalase, yet it may reasonably be supposed that when the same apparatus shows widely diverging amounts of oxygen from different samples of milk, different degrees of catalase content are present. So that, although much of the work cannot be taken as absolutely accurate, it would, I think, be unjustifiable to suppose that no reliance can be placed upon the results, provided the minor degrees of difference are neglected.

Section 3.—The relation of the catalase content to the condition of the milk.

The condition of milk may be affected by the state of the gland before secretion, and by contamination after milking.

The conditions of the gland which show departures from the average normal composition of the milk occur in the early days after parturition, and when the gland is "drying" or is inflamed, as in mastitis.

Bier (1905) showed that the catalase was present in colostrum, but he found the amount variable. Gifthorn(67) (1910) found the content increased in mastitis.

Gerber and Ottiker (1910) found that the catalase content of the milk varied with the breed of the cow, the feeding, and general conditions of all sorts. That it was increased in colostrum, in the milk from a gland when nearly "dry" and in disease of the udder.

Kooper(94) (1911) also found that the breed of cow affected the catalase content.

Spindler(177) (1911) confirmed the work of Gerber and Ottiker, and showed that the catalase content is increased in all inflammatory conditions of the udder, and when the gland is in either the early or late stages of lactation, that is when the gland is not in full activity.

These facts agree closely with those obtained for human milk (*see* pp. 23, 24).

The relationship of the bacterial count has been investigated by many observers, but the concensus of opinion is, that although the catalase content is increased by bacterial contamination, there is no definite relationship between the actual number of bacteria and the catalase, although a rough estimation has been suggested by some observers; this last does not apply to small numbers of bacteria (*cp.* Jensen, p. 18).

Lam(97) (1906) tried to work out a standard of the amount of oxygen which should be evolved by milk, with varying degrees of contamination. He used 10 cc. milk and 5 cc. H_2O_2 (1 per cent.) and considered that normally good milk should give off from 0·3-1·0 cc. O_2, and that if more is evolved the milk is not good. Excess of catalase being given either by stale milk, or by milk obtained from unhealthy cows.

Gerber and Ottiker(66) (1910) gave a higher figure than Lam as being the average amount of oxygen which should be evolved. They used 9 cc. milk and 3 cc. 1 per cent. H_2O_2, and obtained from 2·5-3·0 cc. O_2. More than this amount was probably due to dirt in the milk or to disease of the cow.

Heygendorff and Meurer(78) (1910) and Schroeter(161) (1911) all failed to find any definite relation between the bacteria, and the amount of oxygen evolved.

The irregularity of the figures is well illustrated by some of Schroeter's figures:

Bacterial count.	Sediment figure.	Oxygen given off.
11,775,000	·25	6·5 cc.
12,000,000	·3	2·25 cc.

He thought there was some relation between the sediment and the amount of catalase. This might presumably be either due to leucocytes or to dirt.

The evidence as a whole shows that there is increase of catalase in disease of the gland, or in gross contamination. The estimations of catalase which are carried out in some daries for the detection of these points are apparently only moderately reliable, and of little or no value unless there is a considerable excess of catalase, in which case it may be useful.

On the Presence of Catalase in Human Milk.

Raudnitz(133) (1898) found catalase in human milk, as did also Luzzati and Biolchini(38) (1901).

Marfan and Gillet(109) (1902) obtained an evolution of gas from milk on the addition of hydrogen peroxide.

Friedjung and Hecht(64) (1903) worked upon a large number of samples of human milk. They found the amount of catalase extremely variable; in some samples 2 cc. of milk only gave a trace of oxygen whilst the same amount of other samples gave as much as 4 cc. As a whole the healthier the woman and the freer the milk-flow the less the amount of catalase. Milks of colostral type, rich in cells, give the highest amount of O_2; the time of disappearing of the colostral type of milk after birth was very irregular. The last portion of milk taken from the gland was richer in catalase than the rest. The amount of catalase is increased in some constitutional diseases, and in mastitis, but is very variable. Young mothers have perhaps rather less catalase than older ones, but the number of previous pregnancies seemed to be without influence.

Jolles(84) (1904) found that human milk contained more catalase than cows' milk, but that the amount was very variable, and that one breast may contain more than the other.

Bier(37) (1905) found great differences in the catalase content of the milk of the same woman. It was sometimes absent, and the amounts obtained when it was present was stated to have varied from 3–218 cc. O_2. Colostrum had a higher power of splitting H_2O_2 than normal milk. He states that the blood of a wet-nurse contained about 10 times as much catalase as the milk.

R. van der Velden(199) (1907) carried out a large number of experiments with human milk. The milk was collected by means of a breast-pump, and every precaution was taken to ensure sterility. 5 or 10 cc. milk and either ·5 or 1·0 cc. 30 per cent. H_2O_2 were taken for each experiment. The mixture was shaken for 10 minutes by means of a shaking machine worked by a turbine, the gas being collected in a gas-burette.

The amount of catalase as determined by the oxygen evolved, was found to vary from day to day, and was different in the two breasts at the same time. There was also a great difference between the amounts given by different women. Sometimes there was sufficient catalase to give off 13 cc. O_2 and sometimes only 1 cc., in 24 hours. As a whole, colostrum was richer in catalase than the milk of later lactation periods.

V. d. Velden also tried to ascertain whether there was a relationship between the amount of catalase and of the other constituents of the milk. There appeared to be some degree of connection between the cell-content and the catalase, but little or none between the fat content and catalase. In the fresh carefully-collected milk there was no connection between the bacterial count and the catalase, but if the milk was allowed to stand for 24 hours, then a certain degree of relationship became apparent.

Clinically it was found that the infants of mothers whose milk contained little catalase throve just as well as those whose mothers gave more—nor could any difference be detected between the health of the infants and the daily variation in the catalase content of the same milk.

A. Torday([186]) (1907) thought that catalase in the human milk examined by him, was probably connected with the fat since the cream was richer in catalase than milk alone, and this again than lactoserum.

E. and A. Torday([187]) worked together upon the catalase content of human milk obtained from women in a lying-in ward. The milk was collected with all precautions, and on plating out was nearly always found to be sterile. They found that there was no relationship between the age of the woman, the length of lactation, nor the number of the pregnancies. Pre-natal colostrum was very rich in catalase. As a whole the milk of a weakly woman was richer in catalase than the milk of a healthy one, and in that from a gland which was acting feebly, than in that from one in full working order. They considered the catalase content to be far too variable for it to be any indication of the nutritive value of the milk.

Summarising it appears—

1. That catalase is almost always present in human milk, but the amount varies within very wide limits.
2. That no connection has been traced between the amount of catalase in the milk and the age of the woman, the number of the pregnancy, or of the age of lactation, except in the case of colostrum, where it is increased.
3. That the healthier the woman, as a whole, the less the catalase.
4. The variation in the catalase content does not appear to affect the child in any way.

Catalase is also present in goats' milk, as has been shown by various observers, and it was found by Harden and Lane-Claypon to be present in rather larger quantities in milk collected by catheter, and sterile, than in cows' milk similarly collected.

3. Reductase.

The reductases are bodies whose action consists in bringing about the chemical reduction of a given substance.

Their action is most easily detected if the substance used changes colour as a result of the reduction. For this reason methylene blue has been found satisfactory, and has been largely, although not exclusively, used in the investigation of the presence of these ferments in milk. Methylene blue has been used alone—in which case, if it is reduced, the ferment or body causing such reduction is known as a "*direct reductase.*" It has also been used with formalin and the resulting reduction is said to be brought about by an "*indirect reductase.*" This last body has also been called "aldehyde-catalase," "aldehyde-reductase," "formaldehydase," and it has also been suggested by Bach ([19, 20]) that, following the general type of nomenclature, "redukase" would be more appropriate. Further, inasmuch as the reaction was first discovered by Schardinger(157), it is often known as "Schardinger's reaction," and on the assumption that the body is a ferment it is known as "Schardinger's ferment." These two reactions are most frequently denoted by the letters M.B. (methylene blue) and F.M.B. (formalin-methylene-blue) respectively.

In addition to these two substances some authors have dealt with a third reducing agent, *hydrogenase*, which converts sulphur into sulphuretted hydrogen. This action will be considered after the direct and indirect reductases.

Direct and Indirect Reductases in Cows' Milk.

Vaudin(198) (1897) first showed that milk when fresh was capable of reducing indigo, and that the older the milk the more rapid the reduction; he considered that the rate of reduction depended upon the presence of bacteria.

Neisser and Wechsberg(119) (1900) working upon the reducing power of tissues found that methylene blue was reduced by milk, and they considered that this phenomenon might be used for the determination of the bacterial content of the milk.

Wynter Blyth(207) (1901) showed that fresh milk will reduce litmus.

In 1902 Schardinger(157) published his first paper upon the reducing properties of milk, and it has been the starting point of an immense amount of work by other observers, and of much controversy. Schardinger showed that milk had the property of reducing methylene blue in the presence of formalin; the solution used for this reaction was made up of—

5 cc. saturated solution of methylene blue,
5 cc. formalin,
190 cc. water,

and this solution has been used by all subsequent observers who have worked upon this subject, and is known as Schardinger's reagent or solution.

The solution of methylene blue used by him, was made up of—

5 cc. saturated solution of methylene blue,
195 cc. water,

and this has been used by most subsequent observers, and is generally described as "methylene blue solution."

Schardinger found that when milk was quite fresh the solution which contained formalin was more quickly reduced, while later on, when the milk was older, the reverse was the case. The rate of the reaction apparently depended upon the age of the milk, and the number of bacteria present.

He concluded that one of two things must happen:—

1. Either an aldehyde-like substance was necessary for the reduction of the methylene blue; this was gradually formed in the milk by bacteria, but could be replaced in the early stages by formaldehyde or some other similar substance; or
2. The reaction was due to the "living protoplasm" of the bacteria.

This last he considered most likely to be the true explanation. In this he agreed with Cathcart and Hahn(51), who in the same year showed that many bacteria had reducing properties towards methylene blue, and that the property was probably attached to the protoplasm of the cell.

Schardinger also found that the optimum temperature for the reaction with formalin was 45-50° C., the colour usually disappearing within 30 minutes, after incubation. Temperatures of 20-23° C. were also used.

After the publication of this paper it became generally recognised that the reduction of methylene blue, and of formalin and methylene blue, were different reactions, and inasmuch as Schardinger's reagent was capable of being used in fresh milk the more quickly of the two it seemed that it would be valuable for the differentiation of raw and heated milk.

Utz(189, 190) (1903 and 1904) pointed out that the results obtained with Schardinger's reagent were dependent upon the reaction of the milk. In fresh, slightly alkaline milk it was positive, but in stale milk which is acid in reaction, it was negative, but could be restored even in sour milk by the addition of sufficient alkali. He believed that the lactose of the milk played a part in the reaction.

Siegfeld(171) (1903) also believed the reaction to be unreliable for the detection of raw or boiled milk.

Rullmann(147) (1904) showed that the reaction with Schardinger's reagent was positive even after heating at 68° C. for one hour, but it was negative if the temperature was raised to 71° C. Methylene blue alone was negative at both temperatures. Upon this Utz(193) again wrote emphasising his point about the reaction. Both Rullman and Utz were dealing with the subject solely as a means of differentiating between raw and boiled or heated milk, and there seems to have been some degree of confusion between oxidising and reducing actions, into which it is not necessary to enter.

Schardinger(158) wrote vindicating the reliability of his reaction as a test for boiled milk, and the discussion continued for some time longer upon these lines; the main questions being—

1. Was it a reliable test, or did it lend itself to falsification of milk by the addition of an alkali; and
2. Was it as reliable as the peroxidase reaction (*see* pp. 6, 7), and if so, was it as easy to carry out?

This aspect does not, however, directly concern this report.

The next phase in the investigation of the reductases was now entered upon. It was concerned with the *cause* of the two reactions with methylene blue. Were the two reactions due to the same cause? If so were they due to ferments or to bacterial action? If the former then they might be of biological significance, and if the latter then they might be used as a means of estimating the degree of contamination of the milk.

Smidt(172) (1904) showed that there were three factors in milk which could bring about the reduction of methylene blue; (he appears to have regarded this action as a catalytic one).

These three factors were :—

1. Lactose, or other substances which became alkaline on boiling,
2. Ferments, and
3. Bacteria.

Smidt showed (*a*) that fresh milk a few hours after milking gave a positive reaction with Schardinger's reagent but not with methylene blue alone. The reaction with formalin-methylene blue was weakened by heating to 70° C. and destroyed by heating to 75° C. for 20 minutes. He considered this reaction to be due to a ferment which he called aldehyde-catalase.

The rate of reduction of methylene blue alone, depended upon the number of bacteria present and was in direct relation to it.

He also showed that lactose in alkaline solution at 45-50° C. gives decolourisation in a few minutes and methylene blue is reduced when milk is boiled for about 15 minutes, provided the dye is added at boiling temperature or before boiling.

He therefore considered that the reaction with formalin-methylene-blue was different in origin from that with methylene blue alone.

In effect these views of Smidt's have been almost universally acknowledged to be correct, although a vast amount of labour has been bestowed upon the subject since Smidt's paper appeared.

(In accordance with the practice in most of the work after this period, the terms F.M.B. and M.B. will be used to denote Schardinger's reagent and methylene blue alone respectively.)

Seligmann(165, 166) (1905 and 1906) published several papers dealing with the cause of these two reactions, and attributed both of them to bacteria. Throughout his work, however, I have been unable to find any indication of the age of the milk used, of the quality of the milk and of the precautions taken in collecting it, nor do any bacterial counts appear to have been made.

In some of the experiments antiseptics were used but a germ-free fluid does not appear to have been obtained in any case.

Seligmann found that on the first day after milking only the F.M.B. was reduced, and on the second day both were reduced, whilst finally F.M.B. was sometimes reduced more slowly than M.B. He also found that milk treated with formalin took longer to reduce F.M.B. than raw milk.

These facts he interpreted as showing that formalin reduced the number of bacteria present, and hence increased the length of reduction time. Also he boiled milk for an hour and found that it had completely lost its reducing power, but if inoculated with sour milk, it regained the property of reducing both F.M.B and M.B. and hence both were concluded to be bacterial in origin. The same result was obtained with milk heated to 100° C. and incubated for 24 hours; that is, reduction occurs with both reagents when the surviving spores or bacteria have multiplied.

Seligmann further believed that the reduction of methylene blue was brought about by the decomposition products of casein formed by the action of bacteria, the deficiency of these products in the early hours after milking being made good by formalin, which assisted the reduction of the methylene blue.

He isolated a bacillus which he found had strong reducing powers for F.M.B. and thought that the reduction of F.M.B. by pure fresh milk might be due to the bacteria which had been present in the udder. Seligmann carried out a number of other experiments upon the same lines, but it cannot be said that they are all of them convincing, some of them being capable of an opposite interpretation from that put upon them by him, especially those dealing with the reduction of F.M.B. Evidently the methylene blue in the F.M.B. is capable of reduction by bacteria in the same manner as in the M.B. solution.

Seligmann showed that if milk was centrifuged the body responsible for the reduction of F.M.B. was carried up with the cream, at any rate for the most part, but could not be washed out of the cream. The reduction times obtained were:—

22	minutes for	skim milk.
8	,, ,,	whole milk.
5	,, ,,	cream,

and watery extract of cream did not reduce at all.

He obtained evidence of reduction with precipitated caseinogen, and the reducing substance could not be separated by watery extraction.

Jensen(83) (1906) definitely separated the two reactions with F.M.B and M.B. under the terms direct and indirect reductase.

He showed that a large number of the organisms of milk will produce a direct reductase, the acid-forming organisms bringing about reduction rather more slowly; further that there was no relationship between the reducing and catalytic powers of these organisms, and that therefore these reactions are distinct and due to different properties.

Really pure milk did not reduce M.B. but only F.M.B., and he points out that Seligmann's own work showed the same, as has already been mentioned.

Jensen found that the intensity of the action of F.M.B. depended upon the fat content, the strippings or last portions of the milk reducing much more rapidly than the first milk. He agreed with Seligmann that the reducing body cannot be washed away from the cream and considers that it does not therefore come from the leucocytes.

The following table shows the results obtained by Jensen very clearly, and it also shows that there is no connection between the bacterial content and the reducing power to F.M.B.

Milk.	Bacteria per c.c.	Fat Content. Per cent.	Reduction Time of F.M.B.
1st Experiment:			
First milk	16,000	·55	Not in three hours.
Middle milk...	480	2·70	120 min.
Strippings	360	8·30	15 ,,
2nd Experiment:			
First milk	3,200	1·5	90 ,,
Middle milk	2,800	3·40	75 ,,
Strippings	360	7·80	14 ,,
3rd Experiment:			
First milk	—	1·70	105 ,,
Middle milk...	—	3·35	80 ,,
Strippings	—	6·40	16 ,,

Butterberg(19) (1906) showed that Schardinger's reaction was negative if milk was heated to 70° C. for 20 minutes, and that in all cases where the M.B. reaction was positive, the bacterial count was high.

Brand(44) (1907) found that F.M.B. is reduced best at a temperature of 70° C., but that the reaction becomes negative if the heating is prolonged. M.B. on the other hand is not reduced in fresh milk at 70° C., but is produced in boiled milk if the milk is inoculated with fresh milk and subsequently incubated. It is then reduced at 50° C. but not at 70° C. Brand concludes from these experiments that M.B. and F.M.B. are distinct reactions, of which M.B. is due to bacteria and F.M.B. to a ferment.

As regards the distribution of the ferment, Brand found that although the major part of the ferment goes up with the cream some is left behind in the milk, and will give a positive reaction at 70° C. He also showed that the addition of alkali aids the F.M.B. reaction, and that the reaction can be reproduced in boiled milk by this means. Commercial falsification could, however, be detected by the fact that the boiled control would also be positive.

Monvoisin(112) (1907) also showed that although most of the ferment was precipitated by saturation with magnesium sulphate, yet some remained behind in the filtrate and hence it was not fixed to the fat globules.

Koning(88) (1907) in his admirable studies on milk showed that there are a large number of organisms which, if inoculated into sterile milk and incubated for 24 hours, gave reduction of methylene blue; among these were B. Subtilis, B. Coli Communis, B. Fluorescens Liquefaciens and Non-Liquefaciens, B. Prodigiosus, B. Mesentericus, and some lactic acid producing organism. The time of reduction varied greatly with the different organisms, the conditions of experiment being kept constant.

He also showed that F.M.B. is reduced by sterilised and boiled milk on the addition of lactose or alkali, and that the reduction is more rapid at 100° C. than at 45° C., but is not so rapid as the ordinary reduction with raw milk.

Koning found that colostrum reduced F.M.B. more slowly than ordinary milk, but that pathological milk gave a more rapid reduction. He points out the difference in reduction time between the first milk and the strippings, and gave the following table :—

—	Cow I.	Cow II.	Cow III.	Cow IV.
First milk reduced F.M.B. in ...	30	12	11	42 minutes.
Middle " " ...	6	6	6	42 "
Strippings " " ...	2	4	4	10 "

He found also that heating for one hour at 65° C. was sufficient to destroy the F.M.B. reaction in raw milk. He concludes that the reduction of F.M.B. is due to a specific ferment.

Smidt(173) (1908) again wrote upon the question of the differentiation of the M.B. and F.M.B. reactions. He found, as Seligmann, Brand and Jensen had already done, that the reductase for F.M.B. goes up in the cream on centrifuging, and that it cannot be washed away from the cream. This he thinks is against its bacterial origin, and indeed the table given by Jensen (*see* p. 28) shows very clearly the absence of connection between the reducing power to F.M.B. and bacterial content.

Skim milk incubated for 24 hours gave a shorter reduction time for M.B. than for F.M.B. Also pure, almost bacteria-free, milk if kept on ice at first only gives the F.M.B. reaction, the time of which gradually lengthens as the milk is kept. Subsequently in the course of a few days the M.B. reaction becomes positive, doubtless due to the effect of the formalin in preventing bacterial growth gradually wearing off.

Smidt did not accept Seligmann's idea of the part played by the degradation products of caseinogen in bringing about reduction. Milk as it leaves the udder will only reduce F.M.B., and this is accomplished in about 10 minutes at 45° C. The reducing substance must therefore be present in the udder, and as milk as it leaves the udder is sterile the F.M.B. reaction cannot be due to bacteria. Further, samples of milk treated with chloroform or phenol, and which on plating out were found to be sterile, gave the F.M.B. reaction even after standing for 24 hours.

Seligmann(167) (1908) replied, defending his position, and maintaining that M.B. and F.M.B. were both due to bacteria. He argues that milk which has been kept reduces both reagents in the same time, and if milk be incubated the reduction time for Schardinger's reagent may even be less than for the methylene blue alone; further, that the addition of formalin to milk increases the reduction time in both cases, and that as the effect of the formalin wears off the reduction time for both decreases, thus pointing to the primary increase in the reduction time being brought about by the inhibitory effect of the formalin upon the bacterial growth.

Seligmann's arguments in favour of his views do not seem to be very conclusive, because he does not consider the period at which there is the most marked distinction between the two reactions, namely, in the first few hours after milking. It must also be borne in mind that in the presence of bacteria bringing about the reduction of methylene blue these same organisms would also presumably reduce the methylene blue present in Schardinger's reagent, since the formalin could hardly be considered as inhibiting the bacterial effect during the period of the reduction time.

S. Oppenheimer(128) (1908) obtained the typical F.M.B. reaction in both fresh and stale milk at 70° C. In fresh milk, collected with all possible precautions against bacterial contamination, the reduction of F.M.B. took place in from 10-15 minutes at 50° C. and in 7¼ minutes at 70° C. He found no connection between the rate of reduction of F.M.B. and the bacterial content, but he showed that there was a connection between the fat content and the rate of reduction, since the first part of the milk which was poor in fat had a longer reduction time than the rest of the milk. He considers that the reduction of Schardinger's reagent is due to a ferment.

It seems clear that the reduction of methylene blue alone can fairly be attributed to the unaided action of bacteria, and it is not therefore necessary to continue to bring forward evidence in support of this point; at the same time, since this report is intended to deal with the most important papers upon the whole subject, it seemed advisable to mention briefly the work both of Barthel, P. Sommerfeld and Schroeter which deal chiefly with the reduction of methylene blue and of neutral red respectively by the agency of bacteria in milk.

P. Sommerfeld(176) used neutral red in his experiments upon the reducing power of milk. This reagent gives a strong red reaction with lactic acid and becomes yellow on reduction.

Sommerfeld found (1) that market milk gave reduction in all cases in from 7-20 hours; (2) that in milk collected with all precautions so that the bacterial content was only from 1,000-2,000 per cc. reduction did not occur until the bacterial content had increased considerably; and (3) if milk be heated the reducing power is destroyed, but this returns upon inoculation. With about 200 bacteria per cc. the reduction took 44 hours at 37° C., and with 1,000,000 per cc. about 12 hours at the same temperature. He concluded that the reduction is due entirely to bacteria.

Barthel(24, 25, 26) published three papers (1908, 1910 and 1911) which it will be convenient to take all together at this stage. Essentially he was endeavouring to obtain a measure of the contamination of milk by means of the reduction of methylene blue. He first of all took milk and sterilised it by means of toluol or chloroform, and confirmed the sterility by plating out. This procedure had little or no effect upon the F.M.B. reaction but prevented the reaction with M.B. If, however, acid was added to fresh milk the reduction of F.M.B. was delayed but not that of M.B. Barthel considered that Schardinger's reagent could not be used as a means of testing for contamination.

Summing up the results obtained with M.B., Barthel considered that if milk was of good quality it should not reduce M.B. in less than three hours. If the reduction time was from 1-3 hours the milk was only moderately good, and if the reduction was effected in less than one hour the milk was bad. He found, however, that there was no direct relation between the absolute number of bacteria and the rate of reduction, and that the method with M.B. was more sensitive at a temperature of 38-40° C. than at 45-50° C.

The absence of direct relation between time of reduction and bacterial content is well shown in the table given by Barthel.

which incorporates both his own results and some obtained by Jensen in work which has been already considered.

Acidity.	Decolorisation Time.		Bacteria per c.c.
	Barthel.	Jensen.	
15	9 hours	1 hour	7,300,000
15	5 „	1 „	9,215,000
17	7 „	1 „	5,760,000
15	6 „	1 „	3,840,000
15	6 „	1 „ 10 min.	10,680,000
16	Approx. 7 „	1 „ 10 „	4,450,000
16	„ 6 „	1 „ 20 „	2,590,000
15	„ 9 „	1 „ 45 „	1,860,000
17	„ 9 „	2 „ 30 „	3,710,000
15	„ 7 „	2 „ 30 „	7,190,000

(This table is taken from the paper published in 1911.) The acidity was estimated by Thörner's method.

Schroeter(161) (1911) investigated the relationship between the number of bacteria and the rate of reduction of methylene blue, and was, like Barthel, unable to obtain any direct result. He used 40 cc. of milk and 1 cc. of methylene blue solution at a temperature of 38-40° C. He thinks that if the reduction time exceeds seven hours there will probably have been less than 1-1½ million bacteria per cc., and if reduction is effected in less than two hours there will probably have been more than 1-1½ million bacteria per cc. It appears, therefore, that the degree of contamination by bacteria is not easily measured by this means.

It may perhaps simplify the somewhat complicated details of these two reactions if the position of the matter at the end of 1908 be reviewed and briefly summarised.

It had been shown beyond much possibility of error that milk contains two substances capable of reducing methylene blue. Of these one reduced methylene blue directly, the action being due to the bacteria present in the milk. The other reduced methylene blue only in the presence of formalin, and the action was probably due to a ferment or other substance present in the milk; that this substance was connected with the fat of the milk, but the amount of this substance had no direct relation to the amount of fat present.

After this period the attention of observers was directed to the latter of these two reductions that brought about by Schardinger's reagent. From 1908 up to the present year and even in this year, papers have appeared in very considerable numbers, and a truly large amount of time and labour has been devoted by many observers to this question. It is now necessary to deal with this work in further detail.

Schern(159) (1909) worked upon the presence of the ferment in different stages of lactation. He found that the reaction is frequently negative during the early weeks of lactation, becoming positive later on. This is in accordance with Koning's work on colostrum already referred to (*see* p. 29). The reaction is always positive in cows which have been milked for some months, the

F.M.B. being decolourised in about 10 minutes at 65-70° C. When the reaction is just beginning to appear in the early stages of lactation then a better reaction may sometimes be obtained at 45° C. He also showed that serum has a well marked inhibitory effect upon the reaction. The serum of many animals was examined and the same result obtained with them all.

In this connection it may be mentioned that Gifthorn(67) in 1911 showed that if F.M.B. was decolourised with very great rapidity in fresh milk the milk will have come from a diseased udder, which also agrees with Koning's observations.

Trommsdorff(188) (1909) obtained milk direct from the cow's udder by means of a milking tube. By taking the most stringent precautions against contamination he was able to obtain milk which, on plating out, was found to be sterile. This milk was tested for the presence of Schardinger's reaction and the result was positive. Hence it seems certain that milk which has never been contaminated by bacteria does contain a substance capable of reducing methylene blue in the presence of formaldehyde. Trommsdorff found that the acidity of the milk rose during the course of the reaction, and concluded that this was due to the formation of formic acid from the formalin. He found, however, that the acidity rose in precisely the same manner, whether formalin was added or not, so that it was not due to the production of formic acid.

Kooper(91) (1910) endeavoured to show that the F.M.B. reaction was bacterial in origin. He took milk soon after milking and on the two following days, and using Schardinger's reagent only, found that the reduction time was shortened as the days passed, and that boiled milk regained its reducing power if small quantities of raw milk were added to it. The same remarks apply here which have already been made in regard to Seligmann's work, and it need not be further dwelt upon (*see* p. 30).

Sames(150) (1910) working alone and also in conjunction with Römer(141) brought out many points in connection with this reaction. They investigated the reducing power of the different portions of milk as it leaves the udder. They found, as had in part been done by previous observers, that the first milk very rarely reduced at all, and then only incompletely. Middle milk varied a great deal in its reducing power, and did not always reduce completely; whilst the strippings always reduced very rapidly, never taking longer that 8½ minutes. The various constituents of the three different portions of milk were then most carefully investigated, and it was found that the only constituent which varied markedly in quantity in the different portions was the fat, the content of which was very appreciably higher in the strippings than in the rest of the milk. There was, however, no constant parallelism between the rate of reduction and the percentage of fat. If the cows were kept waiting long between the periods of milking then the reaction was often negative.

As to the relation of the reaction to the age of lactation they were unable to obtain any connection; it was sometimes absent, and when present varied in strengthening the same cow from day to day.

They consider that Schardinger's reaction is essentially due to the activity of the gland, and that the reacting material is derived from the break down of the gland tissues; it can only be considered as due to a ferment if the contents of the gland cells and their excretion products can be called such. Römer and Sames further point out that Heidenhain said that end milk was formed by the break down of cells and Koning (*see* p. 29) showed that pathological milk, which presumably contains cells or their debri, reduced F.M.B. very rapidly. They also point out that this body will not bear dilution, which a ferment will. This substance is destroyed by dialysing for two days, whereas the oxidising ferments of milk are not destroyed by four days dialysis.

The authors also deal with the reduction obtained by Koning at 100° C. This they believed to be brought about by the lactose in the presence of an alkali, a reaction which occurs equally well with boiled milk. If the caseinogen and lactalbumin be removed by the addition of acetic acid or by boiling, the filtrate containing the lactose will promptly reduce F.M.B. on the addition of two drops of $\frac{N}{1}$ NaOH.

Römer and Sames further showed that milk which has been heated or which has never given Schardinger's reaction will do so at once if 0·3 cc. of a one per cent. solution of ferrous sulphate be added; if, however, the ferrous sulphate is boiled either before or after its addition to the milk the reaction is negative. The addition of ferrous sulphate to raw milk accelerates the reaction, but is not the only cause of it, since ferrous sulphate in watery solution does not reduce F.M.B.

Sassenhagen(151) (1910) did not obtain the F.M.B. reaction with colostrum, although he found that traces could be detected in the cream of the later colostrum.

Reinhard and Seibold(137) (1911) collected milk both with a milking tube and also with all aseptic precautions without a milking tube. They investigated the effect of the age of lactation upon the presence of the ferment, as had been done by Schern, and Römer and Sames.

They found that in the early weeks of lactation the reaction was often negative, and that the time of its appearance was very irregular, thus confirming Schern. They also, however, found that in some cases where the reaction appeared negative it could become positive if the cream alone were taken the optimum temperature being 45° C. In cows of from 2-6 months lactation the reaction was always positive, F.M.B. being decolourised in 5-12 minutes at 65° C. and being negative at 70° C. There is no anti-ferment present in the colostrum, for milk and colostrum gives a positive reaction more quickly than milk and water, as was shown by Koning for goats' milk (*see* p. 41). This may be due to a slight adjuvant action, but as Römer and Sames found that the body causing the F.M.B. reaction will not bear dilution, it may also be that the dilution of the control of cows' milk and water is showing slight inhibition.

As regards the distribution of the reducing substance they found that the main part goes up with the cream, and that there is no parallelism between the fat content and the amount of the ferment. There may be much fat and little reducing power, and *vice versâ.* The strippings have the highest reducing power, and the authors agree with Römer and Sames that the reduction of Schardinger's reagent is due to the activity of the gland, and is brought about by a pre-formed enzyme. They showed that the reaction is not influenced by the sucking of the calf, and that different teats yield milk of different reducing power. The reaction is the same whether the milk be collected by milking tube or by milking in the ordinary way, and is not influenced by small numbers of bacteria; an absolutely sterile sample of milk which had never been contaminated by bacteria gave a quicker reduction time than milk containing 46 bacteria per cc. and similar numbers.

Working with milk from diseased udders the authors found that there was much variation in the quantity of the ferment, which varied with the degree of inflammation; if the inflammation was severe the reaction was generally either delayed or absent.

In connection with the question of the activity of the gland, the work of Grimmer(70) (1910) may be mentioned. This observer took glands of cows and of other animals and extracted them with glycerine both before and after grinding with quartz. The glycerine extracts contained no substance capable of reducing either M.B. or F.M.B. Too much stress, however, should not be laid upon this fact since it is possible that the substance is not extracted by glycerine, or it may be that the ferment is only produced during active metabolism.

Rullmann (148, 149) (1911) published two papers upon the mechanism of the Schardinger reaction, and upon the question of its presence in sterile milk.

He showed that cultures of certain bacteria in sterilised milk will give reduction of both M.B. and F.M.B. and that formic acid could replace formaldehyde in the production of the F.M.B. reaction.

In a large number of experiments he showed that if lactose (20 cc. of a 5 per cent. solution) and NaOH (2 mg.) be added to either boiled or sterilised milk that great decrease can be obtained in the reduction time both of M.B. and of F.M.B. The formic acid reagent is also reduced but after a longer time. Lactose alone in watery solution will not reduce F.M.B., but the addition of ammonia, calcium or sodium phosphate to a mixture of lactose and soda brings about a great acceleration in the rate of the reduction, as does also a rise of temperature up to 95° C.

Rullmann also collected milk with a milking tube and obtained many samples of milk which were completely sterile, both broth cultures and agar plates showing no growth. In the sterile samples the tests with M.B. alone were entirely negative whereas the reaction with F.M.B. was positive after a few minutes. (In testing with methylene blue alone the method of Smidt-Müller was used.) In the non-sterile samples methylene blue alone was gradually reduced with increase of the bacterial content. The temperature used for these reactions was 45-50° C. Rullmann found, however, that if milk is heated for prolonged periods to 68-70° C. the reaction is positive or delayed, being negative if the temperature is 70° C. or over. If the milk be heated several times to 100° C. and is kept long enough at that temperature the reaction will be positive.

He concludes that there is a substance present in milk which is capable of bringing about the reduction of Schardinger's reagent at 45-50° C. apart from bacteria, and that this substance is probably a ferment.

That reduction of the same reagent can also be brought about at higher temperature by the action of substances present in milk, *e.g.*, lactose and salts. That these substances probably assist in the reduction of the reagent at the lower temperature.

He also found that the milk from a diseased udder contained more reducing substance for Schardinger's reagent, presumably owing to increased cell-metabolism, bacteria, and blood filtration.

Before leaving the work of the year 1911 it may be of interest to deal very briefly with some work which has been carried out by various observers upon the purely physical aspect of Schardinger's reaction. This in 1910 Bredig and Sommer(45) showed that platinum, iridium, and gold sols, will act in precisely the same manner as the milk ferment, and will reduce F.M.B. in approximately the same time, also that at 70° C. this reaction is greatly accelerated by the addition of alkali.

Formic acid can be used to replace formaldehyde in this reaction, the latter being oxidised almost exclusively to carbon dioxide by platinum sol.

The rate of the reaction depends upon the relative quantities of the aldehyde and of the ferment or sol. If aldehyde is present in excess the rate of reduction is decreased out of all proportion to the amount of the sol present.

Bach(19, 20) (1911) dealing with the mechanism of action of the sols. points out that platinum sol. + hypophosphorous acid (H_3PO_2) + water (H_2O) gave H_3PO_3 (phosphorous acid) + 2H and this, if methylene blue be added, will give reduction, the temperature used being 70° C. The sol. can be replaced by the ferment and the hypophosphite by the aldehyde, according to the work of Bredig and Sommer just quoted.

Bach also showed that milk will reduce nitrates in the presence of acetaldehyde with the formation of nitrates; this reaction will not occur without the addition of the acetaldehyde, unless there is bacterial contamination, in which case reduction of the nitrate to nitrate is readily brought about without further addition.

Acetaldehyde and nitrate together give small quantities of nitrite but the rapidity and degree of the nitrite formation is very greatly increased by the ferment in the milk, and depends upon the amount of the ferment which is present. The optimum temperature is 60° C.

Formaldehyde can replace acetaldehyde but is a much weaker agent.

Bach studied the same reactions with an extract of calves' liver, and found that the liver contained a substance giving similar reactions to those of the ferment found in milk, and presumably identical with it.

As regards the theory of the action Bach suggest the following interesting points: If water contains unsaturated ions of the form $H_2O <$ this may unite with either OH- or H- ions forming labile compounds, viz.;

$$H_2O < \begin{matrix}OH\\OH\end{matrix} \text{ or } H_2O < \begin{matrix}H\\H\end{matrix}$$

Now H_4O is the hypothetical sub-oxide of hydrogen and corresponds to M_4O where M is a metal. The other compound H_4O_3 is hydroperoxydhydrate.

Bach thinks that with platinum a compound $H_2O = O_2Pt$. may be formed by the replacement of the two hydrogen atoms by one of platinum. By this means some of the $H_2O <$ ions would be removed and fresh ones formed so as to restore the original concentration of those ions.

Paal and Gerum(129) had shown that this was the case for Palladium sol. and it seemed therefore probable that this would apply for Platinum.

Bach suggests that the ferment bringing about reduction should be called Redukase, rather than Reductase there being no reason for the insertion of the "t." Further that the particular ferment bringing about the reduction in the presence of aldehyde, should be called "perhydridase."

He considers that there is an analogy between the peroxidase and reductase reactions, viz.:—

Peroxidase + $H_2O = O$ (gives the peroxidase reaction), and
Reductase + $H_2O = 2\,H$ (gives the Schardinger reaction, as in calves' liver).

It seems therefore that the action of the ferment in milk is almost if not entirely identical with that of the sols of the metals.

Before the appearance of the various papers upon this subject in 1911, in conjunction with Dr. Harden I had commenced some experiments upon the presence of ferments in sterile milk. Using milk obtained with a milking tube we obtained the same results as those of the writers previously quoted, namely, an absence of reduction with methylene blue, and the evidence of the presence of a substance reducing F.M.B., although the presence of this substance was somewhat irregular and uncertain.

Since the writing of this report was commenced, Burri and Kursteiner(17) have reconsidered the whole question of the reductases in milk, and have reached conclusions similar to those of previous observers. These investigators did not use sterile milk, and were in consequence obliged to use disinfectants to get rid of the bacteria. They used chiefly phenol, and sodium fluoride. They concluded that there is in milk no substance which reduces methylene blue alone apart from bacteria.

They investigated at some length the question of the formation of reducing substances in milk by boiling, and conclude that such are formed, if the boiling be sufficiently prolonged.

They found that the reduction time of raw milk and of milk which has been just boiled is almost identical, but that the rapidity of the reduction increases with the prolongation of the boiling. The experiments were carried out under anaerobic conditions since the authors found that the length of time of the reduction depended upon the amount of oxygen which was present. For instance if, after boiling or sterilising, the tubes containing the milk for experiment were placed upright and also sloping, the tubes which had been in the sloping position took longer to reduce than the ones which had been in the upright position, presumably because they had had more surface exposed to the air, and had taken up more oxygen. That this was the case the authors proved by the following means.

They took two tubes one small and the other larger, and joined these two together by an opening at one side, to which tubing was fixed. The tubes were closed with india rubber corks through which a glass-tube passed out to pyrogallic acid and potash. The small tubes were for the methylene blue and the larger tubes for the milk. The milk and the methylene blue were put into tubes when all was ready, and in one apparatus the two fluids were mixed; everything used being sterilised. Both apparatus were incubated at 90° C. after having been placed in connection with the pyrogallic acid and potash. The methylene blue in the mixed tube was decolorised in about four minutes, and the methylene blue and the milk in the other apparatus was then mixed, and became white in one minute. The difference was apparently due to the delay in getting rid of the oxygen in the apparatus, in which the mixing of the methylene blue and of the milk had already taken place before incubation.

Using this method the authors carried out many experiments upon the influence of the length of time of heating upon rate of reduction.

In one experiment where no disinfectant was used—

Raw milk gave reduction in		64	minutes.
Milk heated to 60° C. for 10 minutes	reduced in	64	,,
,, ,, ,, 90° C. ,, 10 ,,	,, ,,	60	,,
,, just boiled	,, ,,	60	,,
Sterilised milk	,, ,,	23	,,

Incubation Temperature 38° C.

Further—

Raw milk	reduced in	68	minutes.
Milk just boiled	,, ,,	64	,,
Milk boiled for 5 minutes	,, ,,	56	,,
,, ,, ,, 10 ,,	,, ,,	52	,,
,, ,, ,, 15 ,,	,, ,,	44	,,
,, ,, ,, 20 ,,	,, ,,	35	,,
,, ,, ,, 25 ,,	,, ,,	29	,,
,, ,, ,, 30 ,,	,, ,,	13	,,
,, ,, ,, 60 ,,	,, ,,	7	,,

If different times are allowed to elapse between the first boiling and the testing, it is found that the difference between the reduction times is less marked; the shortening of the reduction time of the raw milk being found to be due to bacterial development and the lengthening of the reduction time of the boiled milk to the taking up of oxygen.

The authors discuss the question of the reduction of the F.M.B. solution, and conclude that it is due to a ferment (for which they suggest the name "formaldehydase"), whose action can be simulated by the addition of alkali or ferrous sulphate. It appears therefore that no appreciable amount of reducing substance is formed in milk by boiling milk, provided the boiling be not prolonged.

Römer(142) (April 1912) wrote again summing up the points which he had shown in 1910 and dealing more especially with the value of Schardinger's reagent as a test for boiled milk. He points out that the reaction can always be made positive (*a*) by the addition of an alkali, which in fact is often done in any case, and (*b*) by the addition of ferrous sulphate in small quantities, as already pointed out by him.

He again remarks that the first milk is negative in reaction, and end milk positive: that raw market milk will often give a negative result and that the reaction cannot be said to be reliable as a test for the previous heating of milk.

Summary of the Causes of the Reduction of Methylene Blue by Cows' Milk.

1. The reduction of methylene blue by milk is due to bacterial contamination.
2. Reduction of methylene blue in the presence of formalin can occur in milk which has been obtained free from bacterial contamination.
3. The reduction of formalin-methylene blue is due to some substance, probably a ferment. This is almost, if not entirely, absent in colostrum, but can usually be found in milk after the early weeks of lactation; it is, however, very irregular in the time of its appearance, and in the quantity present.
4. First milk does not usually contain any of this substance, which is present in the greatest amount in the strippings or end milk; although the fat is the only constituent of the milk which varies greatly in percentage in the various portions (being increased in end milk), and although the ferment can for the most part be removed with the cream, there is no parallelism between the amount of ferment present and the fat content.
5. The action of this ferment can be replaced by alkali or ferrous sulphate, in small quantities.
6. The optimum temperature of reaction is from 45-50° C. the reaction becoming negative at a temperature of about 70° C.

7. The action of this ferment appears to be almost identical with that of the solutions of certain metals.
8. This reaction, apparently due to the presence of the same ferment, can be obtained with an extract of calves' liver, and
9. Reduction of either methylene blue alone or of formalin-methylene blue can be obtained in boiled or sterilised milk, provided the milk be boiled long enough. This reduction seems to be due to the formation of reducing substances, or to the presence of lactose and salts.

On the Presence of Reductases in Human Milk.

The amount of work which has been done upon the reducing substances in human milk is small, but enough has been done to obtain reliable information in this direction. It is much easier to obtain human milk free from any great degree of bacterial contamination than cows' milk, although the amount obtained is small.

Gillet(⁶⁸) (1902) using Abelous and Girard's method of testing for reduction by the conversion of nitrates to nitrites, obtained negative results. It has been shown that Bach obtained similar result with cows' milk (*see* p. 36).

Hecht(⁷⁶) (1904) who worked exclusively with human milk, obtained reduction of methylene blue alone, in from 1-2 days, but he was not sure that his samples were sterile, and does not appear to have controlled them by plating out. The time of reduction is entirely in accord with the reduction by bacteria, and by no other agency. In one case where special precautions were taken to ensure sterility, no reduction was obtained. Reduction was stopped by a temperature of from 60-80° C. Above this temperature, reducing powers again appeared on prolonged heating; presumably due to the lactose.

Hecht does not appear to have used Schardinger's reagent, and his results were most probably due to bacterial growth.

Rullmann(¹⁴⁷) (1904) obtained negative results with Schardinger's reagent, except in one case where he obtained a doubtful reaction.

Smidt(¹⁷²) (1904) who only used one sample of human milk, obtained a negative result.

Koning(⁸⁸) (1907) obtained no reduction of Schardinger's reagent with human milk.

P. Sommerfeld(¹⁷⁶) (1908), using neutral red, obtained no reduction with human milk, except in one case where there was inflammation, and cocci and leucocytes were present in large numbers. Nor did he obtain any reduction using methylene blue alone or in conjunction with formalin.

It appears therefore that *fresh human milk contains no reducing substances.*

On the Reductases in Goats' Milk.

Smidt(¹⁷²) (1904) was unable to obtain any reduction of Schardinger's reagent with goats' milk.

Koning(88) (1907) was also unable to obtain any reduction of F.M.B. with goats' milk, although he thought it might contain a trace of a reducing substance, since mixed goats' milk and cows' milk gave a quicker reaction than cows' milk and water. The same remarks apply here, however, as in Reinhard and Seibold's work (*see* p. 34).

Harden and Lane-Claypon(75) (1912) using milk obtained by milking-tube failed to obtain any reduction either of methylene blue or of formalin-methylene blue, even after many hours.

It appears therefore that *fresh goats' milk contains no reducing substances.*

Hydrogenase in Cows' Milk.

There remains one more substance to consider under the heading of reducing bodies; namely hydrogenase, or that ferment which has the property of forming sulphuretted hydrogen from sulphur.

The literature is not inconsiderable, and the results are quite conclusive. In 1891 Rösing(143), in the course of his researches into the production of H_2S from egg-white, found that if sulphur was added to milk it was sometimes possible to obtain the reaction for sulphuretted hydrogen; the result was, however, sometimes negative.

Raudnitz(135) (1902) in his excellent resumé of the literature deals briefly with the question of hydrogenase, and states that he himself had not been able to detect H_2S in milk, on the addition of sulphur; the same was also stated by Schardinger (1902).

Utz(192) (1903) obtained H_2S from milk on the addition of sulphur after prolonged boiling.

Hoeffter(80) (1904) like Rösing found that fresh milk sometimes gave reduction of sulphur; the negative samples, however, also reduced after they had been incubated for one or two days. The addition of antiseptics prevented this development, and as a result of these and of other experiments he concludes that the development of H_2S in milk upon the addition of sulphur is bacterial in origin.

Jensen(83) (1906) also came to the same conclusion.

Brüning(16) (1906) did not in any case find hydrogenase in fresh milk, but he found that if fresh milk was inoculated with stale milk that H_2S appeared on the addition of sulphur. He found that B. coli was especially active in the production of H_2S from sulphur.

Rullman(149) using milk which was obtained sterile by means of the milking-tube was unable to detect hydrogenase in any case. He concluded that the formation of sulphuretted hydrogen from sulphur was bacterial in origin.

There can therefore be little doubt that the hydrogenase which has been found in raw milk is bacterial in origin.

Summary of the Work on the Reductases and on Hydrogenase in Milk.

1. Direct reductase, and hydrogenase, which have been found in fresh milk, are not present in milk as such, but are due entirely to bacterial contamination.
2. Cows' milk contains a substance, probably a ferment, which reduces methylene blue in the presence of formalin. The presence and amount of this ferment is, however, variable.
3. This substance is absent from human and goats' milk.

4.—On the Presence of Proteolytic Ferments in Cows' Milk.

The presence of ferments which break down proteins into their simpler constitutents has been investigated by several observers. The first authors to deal with the subject were Babcock and Russell, and Vivian.

Babcock and Russell(8, 9) first prepared from milk a solution which had ferment activities. The solution showed the reaction for peroxidase, for catalase, and also had the power of hydrolysing proteins (*c.p.* Neumann-Wender, p. 16).

These activities were believed by the discoverers of this solution to be different manifestations of the same ferment, but they studied the proteolytic action exclusively. They were chiefly concerned with the ripening of cheese, which they believed to be carried out at any rate in great part by the ferment in question. They found that cheese could ripen whilst under the influence of ether, chloroform, &c., and that during the period of observation of such ripening (13-30 days), albumoses and peptones were formed from casein.

In their experiments the milk was carefully collected and was at once treated with an antiseptic, but no initial bacterial count appears to have been made. The authors point out that during the process of ripening the bacterial count decreases and yet the solution of casein continues. The ferment was more active in a neutral or alkaline medium, and was more powerful in the cream than in the rest of the milk, but the degree of activity was very variable, not only in different but in the same species of animal.

The products of activity of the ferment were ammonia, amides, and peptones, resembling (as the authors point out) rather the action of bacteria than of a ferment.

Freudenreich(63) (1900) used milk rendered sterile with ether. In periods up to one month there was frequently no increase in the soluble nitrogen, but if the experiment was continued for longer periods up to 8 months an increase was obtained; the action did not appear to be carried as far as amino acids, and Freudenreich was, therefore, disinclined to believe that it had any part to play in the ripening of cheese.

Boekout and De Vries(39, 40) (1899 and 1901) found that cheese made from pasteurised milk will not ripen, even after the addition of the "milk-ferment." They took milk collected in the ordinary way, and milk collected under all possible precautions against contamination, and subsequently treated aseptically. The soluble nitrogen in this milk increases very slightly during the first few weeks of incubation, but not afterwards. The cheese made from this aseptic milk will not ripen, even though made by an experienced cheesemaker. If the soluble nitrogen be estimated in this form of cheese, and in ordinary cheese it was found that the gain in the former in about 4 weeks was from ·8 to 1·8 cc., and in the latter from 1·9-9·4 cc. of deci-normal alkali. The authors point out that even the aseptic cheese was not entirely free from bacteria, and that the small increase was almost certainly due to these organisms; they conclude, that apart from bacteria there is no proteolytic enzyme in milk.

Spolverini(179) (1902) found evidence of both tryptic and peptic action in all the milks examined by him. The tryptic ferment was rather stronger than the peptic.

Moro(113) (1902) using Mett's tubes was unable to obtain any satisfactory results. He then used a piece of dried fibrin, suspended it in milk, and estimated the amount of fibrin which was dissolved by the milk. Both faintly alkaline and acid media were used. The experiments were carried out over 12 hours at 37° C. The milk was not known to be bacteria-free. He obtained a solution of ·0009 grammes of fibrin in the acid medium, and of ·0019 grammes in the alkaline medium.

Zaitschek(258) (1904) used milk with the addition of acid and both with and without pepsin, also with alkali and with and without trypsin. He tested for the formation of peptone, and in no case was he able to demonstrate the presence of these substances unless pepsin or trypsin had been added. The method he found to be correct to ·005 grammes of protein. It is probable, therefore, that Moro's results are within the limit of experimental error. Zaitschek used human, cows', goats' and other milks.

Snyder(174) (1904) incubated milk and toast at 90° F. for four hours, and found some degree of proteolytic activity, even when chloroform, formalin, or ether was added.

A. J. J. Vandevelde(194, 195, 196) (1907) working both alone and with De Waele and Sugg, investigated the presence of proteolytic ferments in cows' milk. The milk used was not initially sterile, but was sterilised by means of hydrogen peroxide, or acetone-iodoform solution. The latter he considered most satisfactory, and it was exclusively used by him when working alone. Vandevelde carried out a number of detailed experiments, but no estimation of the amount of protein dissolved appears to have been made under five days' incubation. In most cases the amounts calculated were for 20 days' incubation. The amounts of protein dissolved in five days varied from 2 per cent., which was the more usual amount, to 24 per cent. in one instance. Even, however, if it could be shown conclusively that the action was due to a ferment initially present in the milk, the changes which take place even in five days' incubation are more of academic than of biological interest.

ORIGINAL WORK.

It seemed, therefore, necessary to carry out some experiments with milk which had never been contaminated by bacteria. For this purpose I collected milk by means of a milking-tube, and investigated the presence of a proteolytic ferment. All the experiments gave negative results, and as all were carried out upon identical lines, it will suffice to quote two of them.

The milk was plated out at once upon arrival at the laboratory, and the acidity of a further sample estimated. All the apparatus used was sterilised.

Five flasks were taken and into each of them 50 cc. milk was pipetted. One flask was tested at once, as a control; two others were incubated without further addition, and the remaining two received the addition of as much sterilised solid sodium carbonate as was required to bring the total alkalinity up to ·2 per cent. The experimental flasks were then incubated at 37° C. for from 24-26 hours. They were then removed and the protein precipitated with tannic acid solution, 50 cc. being used for each flask. 25 cc. of the filtrate was then taken and the amount of soluble nitrogen estimated by Kjehldahl's method. The same treatment was carried out upon the control flask without the preliminary incubation.

(i.) *Bacterial count 23 per cc. (Cow troublesome and kicked out the catheter.) Incubation time, 25 hours.*

Sample used.	Soluble Nitrogen in cc. $\frac{N}{10}$ solution.
1. Control, 50 cc. milk	3·6
2. 50 cc. milk incubated	3·6
3. 50 cc. milk ,, ...	3·4
4. 50 cc. milk alkali	3·7
5. 50 cc. milk ,, ...	3·7

(ii.) *Bacterial count 3 per cc. Incubation time 26 hours.*

Sample used.	Soluble Nitrogen in cc. $\frac{N}{10}$ solution.
1. Control, 50 cc. milk	3·75
2. 50 cc. milk incubated	3·7
3. 50 cc. milk ,, ...	3·7
4. 50 cc. milk alkali	3·65
5. 50 cc. milk ,, ...	3·4

It seems, therefore, that initially sterile milk does not contain any ferment acting upon the proteins of milk either in the normal reaction of the milk or in an alkaline medium.

On the Presence of a Proteolytic Ferment in Human Milk.

Spolverini (1902) found both tryptic and peptic ferment action in human milk, and Moro (1902) using the method already described (*see* p. 43), found that ·0006 grammes of fibrin was dissolved by human milk in 12 hours in the acid solution, and ·0013 grammes in the alkaline sample.

Friedjung and Hecht (1903) using gelatine tubes endeavoured to detect the presence of proteolytic ferments in milk with a view to establishing a relationship between the activity of this ferment and of catalase. The milk was not bacteria-free, and thymol was added. The tubes were incubated for three days, and at the end of that time about 2-3 mm. of gelatine had usually been dissolved; the catalase content differed as much as 8-fold in the sample examined, and the authors conclude that "The proteolytic ferment is not only of too slight activity, but its intensity of action shows too great difference for any relationship to obtain between it and catalase."

Zaitschek (1904) using the method already described (*see* p. 43), was unable to detect any proteolytic action in human milk, of either the peptic or the tryptic variety.

Austin(6) (1908) working upon the milk of 21 women in different states of health, was quite unable to trace any proteolytic enzyme in human milk.

He used Volhard's method, and the soluble nitrogen was estimated by Kjehldahl's process. No hydrolysis of protein could be detected even after 14-16 days. Austin concludes (1) There is no evidence of auto-digestion of human milk, at least under the conditions pertaining to such digestion in organ tissues. (2) The digestion disturbances of infants fed upon human milk can have no relation to such an enzyme, as the milk of both healthy and sick women was examined.

Summary of the Results upon the Presence of Proteolytic Enzymes in Human and Cows' Milk.

(1) In initially sterile cows' milk there is no proteolytic ferment.

(2) There is no proteolytic ferment present in human milk.

On the Presence of a Peptase in Human and Cows' Milk.

Wohlgemuth and Strich(205) (1910) working on both human and cows' milk as well as that of other animals, found evidence of a ferment in milk which will hydrolyse glycyl-tryptophane. Warfield(207) (1911) working upon human milk only, found the same. These authors did not work with initially sterile milk, but treated the milk with antiseptics. In view of this fact, and of the small amount of evidence, the presence of this possible ferment will not be further considered.

On the Presence of "Fibrin-Ferment" in Human and Cows' Milk.

Schlossmann(160) (1902), Camerer([illegible]) (1901), and Moro and Hamburger(116) (1902) found that if human milk was added to hydrocoele fluid, that coagulation took place. Bernheim-Karrer([illegible]) showed that this was also the case when cows' milk was added to hydrocele fluid. It was supposed that since fibrinogen was present in hydrocele fluid, that "fibrin-ferment" must be present in the milk. Moro, Camerer (who used blood), and Bernheim-Karrer all found, however, that the reaction was little, if at all, influenced by heat. It is, therefore, extremely doubtful if the substance bringing about this action can be considered to be a ferment. In view of this fact, and of the extremely complicated nature of blood-clotting, this reaction will not be considered any further.

(5) LIPOLYTIC FERMENTS.

(A) ON THE PRESENCE OF LIPASE IN COWS' MILK.

Spolverini, Luzzati, and Biolchini (1902), also Marfan and Gillet (1902), found that cows' milk was capable of splitting monobutyrin into butyric acid and glycerine. The action Marfan and Gillet found to be specific for this substance; oil was not acted upon at all, nor other compounds of butyrin.

Moro (1902) found that cows' milk had the power of splitting olive oil. The method used by him is not, however, sufficiently accurate to be convincing. The presence of fatty acid was tested for by the formation of an emulsion when alkali was added.

Leperre(101) (1904) was unable to trace any decrease in fat content even after several weeks, the other constituents of the milk being, however, altered in quantity.

A. J. J. Vandevelde (1907) determined the acidity of the milk as a test for the formation of fatty acids. The milk was treated with the acetone-iodoform mixture used by him, and after incubation was distilled. No change in the acidity of the distillate could be detected even after several weeks.

On the Presence of Lipase in Human Milk.

Spolverini, Luzzati, and Biolchini, also Marfan and Gillet, all found monobutyrinase in human milk.

Friedjung and Hecht (1903) also found the same, but did not investigate the presence of an autolytic lipase.

Hippius(79) (1905) using Mankowsky's reagent for the detection of the presence of fatty acids, believed that human milk was capable of splitting olive oil. This reaction was destroyed by heating to 64° C.

None of the investigations connected with the presence of lipase or of monobutyrinase were carried out upon milk collected free from bacteria. This throws the results open to much suspicion. The absence of an autolytic lipase may, however, be assumed to be reliable, and is the only one to which any value need be attributed.

The difficulty of obtaining human milk, and the still greater difficulty of obtaining a reliable method for the investigation of the presence of autolytic lipase, prevented me from investigating the presence or absence of such a ferment in human milk.

The small amount of data upon the presence of mono-butyrinase, and the extremely problematic value of such a ferment to the infant, even if it were found to be present in milk collected free from bacterial contamination, render it unnecessary to dwell any further upon this subject.

(B) On the Presence of a Salol-Splitting Ferment in Cows' Milk and in Human Milk.

The literature of the ferments in milk includes quite a number of papers dealing with the presence in milk of a ferment having the property of splitting salol into phenol and salicylic acid. The presence of this ferment is tested for by adding salol to milk, incubating, and then testing for the presence of phenol and salicylic acid by the addition of ferric chloride.

Nobecourt and Merklen(123) (1901) first pointed out the presence of this reaction, which was positive in human milk but negative in cows' milk. They also found that it was present in the serum of nursing women, but was absent in the urine.

Spolverini (1901) and Luzzati and Biolchini (1902) also found this ferment present in the milk of women but not of cows.

Moro (1902) showed that no reaction appears under 12 hours' incubation at 38° C. Further, that the reaction is not affected by boiling, which is against its being due to a ferment.

The reaction was positive in all samples of human milk examined by him but always negative in cows' milk.

Desmoulières(54) (1903) showed that certain salt solutions which are neutral to litmus and phenol-phthalein will split salol in precisely the same manner as the supposed ferment, and that the reaction occurs after boiling; he thinks it must be due to the alkaline phosphates in the milk.

Miele and Willem(111) (1903) obtained evidence of the presence of salicylic acid in an alkaline solution of salol after incubation for 24 hours at 37° C. They found that the reaction was given by cows' milk and by human milk both raw and boiled, by saliva and by pancreatin, if the reaction of the medium was faintly alkaline.

Desmoulières(54) (1903) again wrote pointing out that he had already dealt with the question of the reaction of the medium, and had also pointed out the probable sources of error in Nobécourt and Merklen's work.

Friedjung and Hecht (1903) working with human milk found salolase present.

Pozzi-Escot(132) (1903) showed that the lipase derived from castor oil beans which will attack fatty acid compounds of salicylic acid will not touch the phenol compounds.

A. J. J. Vandevelde(196) (1907) examined the question of the presence of salolase in cows' milk. He used acetone-iodoform as a disinfectant, and first showed that neither the reagent nor the reaction of the milk was able to produce a splitting of salol. He then took both raw and boiled cows' milk and after adding the disinfectant placed the flasks in the incubator. After 25 days he was able to demonstrate that the raw milk contained salicylic acid of a strength of about ·0001 per cent., and he believed this action was due to a ferment present in the milk.

Grimmer (1910) in his work upon the ferments present in the glands themselves, found salolase present in all the glands examined except in the case of a lactating cow.

Rullmann (1911) using initially sterile cows' milk was quite unable to detect any salolase in the sterile samples examined by him. He concludes therefore that salolase when found in milk is of bacterial origin.

If the results obtained by the various observers be considered for a moment it appears fairly evident that there is no ferment present in cows' milk which can split salol. The only observers who have found it present in any quantity have also found it present in the boiled samples. This is much against the reaction being due to a ferment at all. Nor can any stress be laid upon the minute quantity of salicylic acid produced in Vandevelde's experiments after so long a period as 25 days.

As regards human milk it appears that there may be a body present which is capable of splitting salol, but inasmuch as this action has been found to take place in boiled milk it is doubtful whether it is of ferment nature.

Summary of the Results upon the Presence of Salolase.

1. It appears that cows' milk does not contain any substance which splits salol.
2. Human milk seems to have the power of splitting salol, but it is doubtful whether this action can be attributed to a ferment.

(6).—ON THE PRESENCE OF LACTASE AND GLYCOLYTIC FERMENTS IN MILK.

Spolverini (1902) found evidence of glycolysis present in all the milks examined by him; Zaitschek, however (1904), found no change in reducing power on incubation, except in the case of bacterial contamination, and he hence considered that Spolverini's results must have been due to this cause.

Stocklasa(182) (1904) prepared a ferment solution from milk by means of alcoholic precipitation, which he considered had lactose-splitting power. The milk used was not initially sterile, but was preserved by means of antiseptics. 50 cc. of a 40 per cent. solution of lactose was used for each experiment, and the amount of lactose lost varied from ·32—·68 grammes. The experiments were carried out over from 3-5 days, at 37° C.

A. J. J. Vandevelde(197) (1908) believed that he had evidence of the presence in milk of a glycolytic ferment. The milk was not initially sterile, but was disinfected by means of iodoform and acetone. The amount of lactose present was estimated both by the polarimeter and by Fehling's method. There was no increase in reducing power, so that the lactose was not split into dextrose and galactose. The fluids used were found to be bacterial-free as a result of the addition of the disinfecting agent. Vandevelde carried his experiments over very prolonged periods, and the amount of sugar lost, even after many months, was very inconsiderable, and only occurred in the acetone-iodoform samples.

The samples preserved with formol showed no change, or so little as to be negligible. Thus—

—	After 27 days.	After 155 days.	After 429 days.
	Lactose per cent.	Lactose per cent.	Lactose per cent.
Milk sterilised by heat ...	4·63	4·76	4·50
Milk and formol	4· 3	4·63	4·31
Milk and acet-idod	4·06	2·22	1·86

In another case, after treatment with antiseptics, there was a decrease of 0·362 per cent. of lactose in 3 years and 5 months.

This work of Stocklasa's and Vandevelde's is open to the objection that the milk was not obtained free from bacterial contamination, but was only rendered sterile afterwards. The varieties of bacteria which will ferment lactose are very numerous, and it is, therefore, not unlikely that traces of ferment action might occur in the solution in presence of the bacteria killed by means of the antiseptics.

The only value that lactase in milk could have for the infant, must consist in the ferment acting with some rapidity upon the lactose within the first few hours. For this purpose the 27 days of Vandevelde's experiment are of no significance, especially in view of the very small amount of sugar decomposed.

It was shown by Aders Plimmer(131) (1906) that lactase is present in the alimentary canal at birth, and in some animals just before birth, in considerable amount, compared with which the strength of ferment obtained in milk by Stocklasa and Vandevelde, fades into complete insignificance. Various authors have also detected lactase in the alimentary canal of new-born infants (*cp.* Ibrahim). Even if a ferment of the strength found by these observers is present in milk collected free from bacterial contamination, it is difficult to believe that it could have any biological value whatever. For this reason it will not be considered any further.

(7).—AMYLASE.

The term amylase is applied to a ferment which acts upon starch or amylum, breaking it down into simpler substances, namely, dextrins or sugars.

The fact that starch could be broken down into sugar by the action of ferments has been known for a very long time; as early as 1833 Payen and Persoz prepared a solution from malt extracts which had this power. The substance bringing about this result they termed "diastase."

The work of Fraenckel and Hamburger([illegible]) in 1906 renders it probable that this substance "diastase" consists of two ferments; one of these liquefies starch, converting it into dextrin, and is hence a true amylase, whilst the other breaks the dextrin down into sugar (maltose), and is, therefore, a "dextrinase." When maltose is further converted into glucose, the action of a third ferment, maltase, is required. (*Cp. also* Duclaux([illegible]).)

It will be shown in the following pages that the presence of amylase alone appears to have been demonstrated in milk, since most observers have traced the conversion of starch to the dextrin stage only; it may, however, be pointed out that the difficulties of tracing minute quantities of sugar, either maltose or dextrose in milk, is enormous, owing to the presence of the lactose. The evidence of most investigators is in favour of the starch not being split beyond the stage of dextrin.

AMYLASE IN COWS' MILK AND HUMAN MILK.

Lépine([102]) (1870) demonstrated the presence of a ferment capable of splitting starch in the large number of tissues examined by him, with the exception of the crystalline lens.

Bécamp([28]) (1882) prepared a ferment solution from milk which had the property of breaking down starch into simpler products. This ferment he called "galactozymase." The action was much stronger in human milk than in cows' milk.

Moro([113, 114, 115]) (1898, 1900 and 1902) was led to the investigation of the presence of amylase by studying the ferment content of infants' stools.

He found that the stools of breast-fed babies contained more diastase than those of babies artificially fed. All the stools showed the presence of the ferment, derived apparently from the alimentary canal, but the stools of the breast-fed babies had a higher content, presumably owing to the presence of the ferment in the milk taken by the mouth.

Moro isolated the protein from human milk with alcohol, and subsequently extracted the precipitate with ether. The residue was dried in vacuo and found to possess definite amylolytic power. Traces of maltose were found, but the starch had for the most part not been hydrolysed beyond the stage of dextrin. In cows' milk, however, the result was negative, using the same method. These results were confirmed by Luzzati and Biochini (1902), who found amylase in human milk and traces of it in asses' milk, but none in cows' milk or goats' milk.

Nobécourt and Sévin([124]) found amylase in the milk of nursing women, and also investigated the presence of amylase in the blood; this will be dealt with later.

Spolverini (1902) found amylase in human and dogs' milk, but was unable to detect any in cows' milk. His well-known feeding experiments will be dealt with later. (*See* p. 56.)

Friedjung and Hecht (1903), working with human milk, found amylase present in all the samples examined by them.

Zaitschek (1904) worked with human and cows' milk, as well as with the milk of other animals, and found evidence of amylolytic ferments in all samples examined. Known quantities of starch were added to the milk, also thymol or toluol, and the samples were incubated for 24 hours.

The amount of starch hydrolysed was estimated by weighing the increase of reducing sugar formed during incubation; the sugar was estimated by weighing the copper oxide after the addition of Fehling's solution. They found increase of copper oxide in all samples, but in varying amounts. The sugar was calculated as maltose, and 100 cc. cows' milk gave from 0·1-0·4 grammes increase of maltose, whilst human milk gave from 0·1-0·9 grammes increase.

It may be pointed out that these results are somewhat different from those of other observers, in that hydrolysis of the starch was carried a stage further, namely to maltose, instead of to dextrin, as has been found by most observers.

Hippius (1905) also found amylase present in human milk. The ferment will bear a temperature of 65°-69° C., but is inactivated at 70° C.

Koning (1906) in his work upon milk discusses the question of the presence of amylase both in milk and in plants, &c. He showed that certain bacteria were capable of producing amylase, notably B. Mesentericus, B. Subtilis, B. Fluorescens Liquefaciens, and Non-Liquefaciens. He found amylase constantly present in cows' milk which had been carefully collected, and did not think it was due to bacterial contamination. He tried several methods of investigation, and finally used the following:—

10 cc. milk collected with great care were put into a test-tube with a few drops of soluble starch solution, of known strength. This was then incubated, and after varying times iodine (Iodine, 1 part, Potassium Iodide, 2 parts, and water, 300 parts) was added. Small amounts of amylase were always found; about ·015-·02 grammes of starch were decomposed by 100 cc. milk in half hour. Any increase in this amount was pathological. The first and middle milks were richer in amylase than the strippings. The ferment was destroyed by 45 minutes at 68° C.

Giffhorn(67) (1910) using mixed cows' milk found that 100 grammes of milk will decompose from ·01-·25 grammes of starch. The amount of amylase is increased in pathological conditions of the udder. It is killed by 30 minutes at 65° C.

Wohlgemuth and Strich(205) (1910) found diastase present in large quantities in human milk, especially in the early days of lactation.

They were unable to detect amylase in either goats' or cows' milk.

Their experiments in regard to the source of the ferment will be returned to later. (*See* p. 56.)

There is, therefore, an accumulation of evidence in favour of the presence of amylase in *human* milk, Koning and Zaitschek alone being able to detect the presence of this ferment in *cows'* milk.

ORIGINAL WORK.

To assist in determining this point, I undertook some experiments upon cows' milk collected by milking-tube, the bacterial count being either nil or negligible.

The method used was that of Koning, and the results were positive on all occasions. It will suffice to quote two experiments since the routine followed was precisely the same in all. The starch solution used was a 1 per cent. solution of soluble starch, which was made fresh, and sterilised before using. After incubating for the times stated, iodine solution as prescribed by Koning was added to each tube. The boiled controls were always negative, but several controls were always made in order to have varying colour standards for comparison with the ferment-containing tubes, if necessary. In the tubes from which the starch had disappeared a brown colour was struck immediately upon the addition of the iodine, which gradually faded to white, presumably owing to the taking up of the iodine by the protein and other substances present in the milk, which are known to act in this way. The controls gave the characteristic blue iodide-of-starch coloration. The temperature of incubation was 37° C.

The results can be tabulated, and are shown in the following table.

Experiment 1.—*Bacterial count,* 1 *per cc. Incubation time,* 3 *hours at* 37° *C.*

Milk.	Starch solution used =	Grammes of Starch.	Result with Iodine.
10 c.c.	·03 c.c.	·0003	Brown.
,,	·05 ,,	·0005	,,
,,	·1 ,,	·001	,,
,,	·2 ,,	·002	,,
,,	·3 ,,	·003	Blue-gray.
,, (boiled)	·03 ,,	·003	Blue.
,, ,,	·1 ,,	·001	,,

Experiment 2.—*Bacterial count,* 3 *per cc. Incubation time,* 3½ hours at 37° *C.*

Milk.	Starch solution used =	Grammes of Starch.	Result with Iodine.
10 c.c.	·1 c.c.	·001	Brown.
,,	·2 ,,	·002	Faintly gray blue.
,,	·3 ,,	·003	Blue.
,,	·4 ,,	·004	,,
,, (boiled)	·1 ,,	·001	,,
,, ,,	·2 ,,	·002	,,

It seems, therefore, certain that cows' milk does contain small quantities of amylase, 10 cc. milk being able to split from ·001-·002 grammes of starch, in 3 hours at 37° C.

Summary of the Work on Amylase in Cows' Milk and Human Milk.

The evidence shows that—

(1) Amylase is present in human milk, in all the cases examined.

(2) That it is present in small quantities in cows' milk, but has frequently not been detected.

(3) The ferment hydrolyses starch to dextrin, and is hence a true amylase,

Division B.—SUMMARY OF THE RESULTS OBTAINED IN DIVISION A.

The work dealt with in the preceding sections shows that—

1. Peroxidase is constantly present in cows' milk, but is very inconstant in its appearance in human milk. The reaction for the detection of this ferment is, on the whole, more marked in colostrum, or in mastitis milk. (*cp.* Weber, Gifthorn, Marfan and Gillet, Friedjung and Hecht, and Kastle and Porch.)
2. The ferment comes down with the albumin fraction; it is most probably derived from broken-down cell-tissue, in the case of human milk probably from the leucocytes.
3. The reaction is believed to be dependent for its production upon the presence of a metallic compound (iron or manganese) in colloidal state; it is destroyed by acid, by any excess of alkali, or by digestion.
4. Catalase is present in small amounts in milk collected initially free from bacteria both in cows' milk and in human milk.

   The amount is increased in colostrum or in mastitis milk, or, in women's milk from badly acting glands.
5. In ordinary milk the major part of the catalase is derived from the contained bacteria.
6. Direct reductase is derived exclusively from the contained bacteria in milk, whether cows' milk or human milk.
7. Indirect reductase, or formaldehydase, is present in sterile cows' milk, which has never been contaminated by bacteria; it is most frequently absent in colostrum. It has some connection with the fat of the milk.
8. Indirect reductase is absent in human milk.
9. The hydrogenase said to have been found in cows' milk is of bacterial origin.
10. Proteolytic ferments are not present in milk collected free from bacteria in either cows' or human milk. A peptase has been described in milk not known to be sterile, but this has not hitherto been confirmed.
11. Lipase is not present in cows' milk. A ferment, monobutyrinase, is stated to be present in both human and cows' milk, the milk not being known to be initially sterile.
12. Salolase has not been found in cows' milk collected free from bacteria; it is stated to be constantly present in human milk (bacterial content not given).
13. Traces of lactase and of glycolytic ferment have been found in cows' milk not collected free from bacterial contamination.
14. Amylase is present in small amounts in cows' milk collected free from bacteria. It is also constantly present in human milk.

The results obtained in the preceding part of this work may be tabulated as follows:—

Name of Ferment.	Presence in pure Cows' Milk.	Presence in pure Human Milk.	Of Bacterial Origin.
Peroxidase	Constantly present	Inconstant	—
Catalase	Present	Present	In great part
Reductase—direct ...	Absent	Absent	Yes
Reductase—indirect ... (formaldehydase).	Present	Absent	No
Hydrogenase... ...	Absent	No data	Yes
Proteolytic	Absent	Absent	Yes
Peptase	Present (?)	Present (?)	(?)
Lipase	Absent	No data	—
Monobutyrinase ...	Present (?)	Present (?)	(?)
Salolase	Absent	Present (?)	(?)
Lactase and glycolytic	(?)	No data	—
Amylase	Present	Present	—

The ferments known to be present in pure cows' milk are catalase, peroxidase, formaldehydase, and amylase.

Those known to be present in human milk are catalase, amylase, sometimes peroxidase, and possibly salolase, although this has not been investigated in milk known to have been sterile.

In the case of the others mentioned the evidence is scanty, and the work has not been carried out in initially sterile milk.

Division C.—ON THE BIOLOGICAL VALUE OF THE FERMENTS PRESENT IN COWS' MILK AND IN HUMAN MILK, IN THE FEEDING OF INFANTS.

It has been shown in the preceding part of this report that the ferments which are present in cows' milk, apart from the action of the contained bacteria are, catalase, peroxidase, formaldehydase, and amylase.

Further monobutyrinase, glycyl-tryptophanase, and glycolytic ferments have been found in milk not initially sterile.

Those ferments which are found in pure human milk are catalase, amylase, and sometimes peroxidase; whilst salolase, monobutyrinase, glycyl-tryptophanase have been found in milk not known to be initially sterile.

These ferments can be grouped under three headings:—

1. Those acting upon substances present in the milk, and presumably concerned in digestion.
2. Those acting upon substances not known to be normally present in milk.
3. Those concerned with oxidising or reducing processes.

Under the *first* heading the trace of lactase found in milk not initially sterile alone appears, and as this subject has already been dealt with in some detail, it need not be considered any further here. (*See* p. 47.)

Under the *second* heading appear amylase, monobutyrinase, glycyl-tryptophanase, and the salolase in human milk. These last three ferments have not been shown to be present in initially sterile milk; the literature of the ferments shows that ferments are specific in their action, and there is no reason to suppose that these ferments (if present in uncontaminated milk) would act upon any other substances than those from which they derive their names. As these substances are not known to be present in either human or cows' milk, it is hardly possible to suppose that the presence in milk of the ferments which act upon them, can have any biological significance.

Formaldehydase also strictly comes under this heading. It is not, however, at all certain that this action is dependent for its production upon the presence of a ferment, since it has been shown that it can be restored after removal by heat by either alkali or ferrous sulphate. As, however, it is entirely absent in human milk, it may reasonably be assumed that it is without biological value to the infant.

Amylase remains to be considered. It is present in cows' milk in amounts too small to split any appreciable quantity of starch, but is stated to be present in somewhat larger amounts in human milk. Clinical experience has definitely shown that starch is an unsuitable food for infants during the first few months of life. Further, it has been shown that amylase is present in the saliva and pancreatic juice of new-born infants (*cp.* Zweifel(219), Moro(114), Ibrahim(81)), so that the amylase of the milk is not in any case required for the digestion of starch, even were such digestion necessary in the early months. It is not necessary to seek further for any possible function for the amylase of milk.

Under the *third* and last heading catalase and peroxidase remain to be considered. It has been shown that catalase is present in small amounts in all tissue extracts, so that its absence in milk, rather than its presence, would be cause for inquiry. As a whole the healthier the gland the less the catalase, the amount being greater in colostrum or mastitis-milk.

The irregularity of the appearance of peroxidase in human milk would alone be a strong argument against attributing any value to this ferment in the general nutrition of the infant. Peroxidase is, moreover, destroyed by acid, and by digestion, so that its life as a ferment must be ended on reaching the stomach.

Clinical evidence has also shown that no difference can be detected between infants, the milk of whose mothers contains varying amounts of these ferments, or even no peroxidase.

Peroxidase, catalase, and amylase are found in considerable amounts in the blood, and it is only consonant with general physiological experience to suppose that the small and varying amounts of these ferments which are found in milk are derived from the blood-stream by filtration.

The presence of amylase in the blood has been known for many years, and was found by Achard and Clerc([illegible]) (1901) to decrease in certain disease (*cp. also* Lepine(102) and Bial(36)). Nobécourt and Sévin(125, 126) (1902) showed that the amylase content of the serum of wet-nurses and of cows varied very considerably. Amylase was present in both urine and milk of the wet-nurses, and in the urine of cows, but they failed to detect it in cows' milk, presumably owing to the small amount present. The amylase content of the urine was always higher than that of the milk. They were unable to detect any difference in the amylase content of the blood of breast-fed and artificially-fed infants.

The presence of catalase and peroxidase in the blood is so generally known that no evidence need be adduced in this connection. Senter(169), Kastle and Amoss(85) v. Itallie(82) and many others may be compared.

It appears that the ferment content of the blood can be altered by various conditions. Thus Dubourg(55) (1889) found that both in himself and in rabbits the amylase content of the urine was increased on a diet rich in starch.

Spolverini(179, 180) (1902) fed lactating animals upon food containing ferments other than those normally present in the milk of that species, and obtained evidence of the presence of these foreign ferments in the milk.

Landtsheer and Vandevelde(98) (1903) did not confirm Spolverini's results, but Spolverini wrote pointing out that his procedure had not been followed, and that the criticisms were not justified. (Arch. de méd. des Enfants, 1904.)

Wohlgemuth and Strich(205) (1910) tied the pancreatic duct of a bitch in full lactation, and found that the amylase content of blood and urine, and subsequently of the milk, increased. The blood content was always higher than that of the milk, but that both fell together.

It seems that ferments can pass from the blood into the milk, and that this can account for the presence in the milk of ferments identical with those in the blood, more especially as the amount of these ferments tends to increase when, owing to disease of the gland, and inflammation, the exudation of serum is increased in amount. Some portion of the ferment content is, however, probably derived from the leucocytes of the milk and from the breaking-down of the cells of the gland itself in the course of its metabolism.

As a result of the survey of the available data upon the ferments present both in human and in cows' milk, there appears to be no ground for claiming any biological value for these substances.

PART III.

ON THE SO-CALLED "PROTECTIVE SUBSTANCES" IN MILK.

In this part of the report no attempt is made to deal fully with the whole question of the protective substances in milk. The field has been purposely strictly narrowed to include only those substances which have been shown to be present either by direct investigation, or by feeding. Any attempt to deal with the biology of milk in relation to the antigens, whose nature is determined by injection, leads at once into the most subtle and complex branches of biological research. This has purposely been omitted, so as to concentrate as closely as possible upon the bearing of the question on the feeding of infants. Further such side issues as necessarily arise have been treated very briefly, without attempting to discuss the literature, again in the endeavour to avoid complication of the main issue.

The term "protective substance" has been applied to certain bodies in the blood which have been shown by experiment and subsequent practice to afford protection, and even immunity, to the organism from certain diseases. It does not, however, necessarily follow, nor can it be assumed *a priori* that, should these substances be found in milk, they subserve the same function as when present in the blood-stream.

No original work has been carried out for this part of the report, and no attempt will be made to deal with the basis of our present knowledge of the bodies concerned in the production of this immunity; an elementary acquaintance with the fundamentals of this branch of knowledge will be assumed. The presence of such bodies, and their possible value to the infant as far as is known from the work at present published will be considered.

DIVISION A.—ON THE PRESENCE OF "PROTECTIVE SUBSTANCES" IN MILK.

These substances may for the present purpose be conveniently considered under five headings according to their known action.

1. Those concerned with hœmolytic action.
2. Those concerned with bactericidal action.
3. Those bringing about the precipitation of certain substances (precipitins).
4. Those bringing about agglutination of bacteria (agglutinins).
5. Antitoxins.

1.—*The substances concerned in hoemolytic action.*

Three bodies are necessary for hœmolysis to occur; the red corpuscle, the amboceptor or immune body, and complement. Further for such a series of bodies to form a hœmolytic system the red corpuscles used must be such that the amboceptor is capable of sensitising the red corpuscle and thereby rendering it susceptible to the action of the complement. Each variety of corpuscles requires its own amboceptor, which is specific for that particular variety, and it is therefore evident that there are a large number of bodies functioning as hœmolytic amboceptors for the blood of different animals.

It has now to be considered whether there is evidence of the presence of either amboceptor or complement or both, in milk, and cows' milk will be dealt with first.

The hœmolytic factors of cows' milk.

If cows' milk contained both amboceptor and complement hœmolysis would occur on the addition to the milk of a suspension of suitable red corpuscles. This however has not been found to occur by any observer using average milk. In 1907 Pfaundler and Moro(274) obtained evidence of the presence of complement in cows' milk by using the system.

Guinea-pigs corpuscles (·2 c.c. 5 per cent.) + inactive ox-serum (·05 c.c.).
+ raw milk (·5 c.c.).

The system guinea-pigs corpuscles + inactive ox-serum + ox complement being a suitable one. Using the same system in the following year I(256) also obtained evidence of the presence of complement in cows' milk. The subject was discussed at a meeting of German pediatricians, when Noeggerath and Bauer both stated that they had been unable to obtain hœmolysis after the method of Pfaundler and Moro, except in colostrum or mastitis-milk. Noeggerath(273), however, subsequently wrote saying that Moro had demonstrated to his and Bauer's satisfaction that hœmolysis could be obtained in milk from a healthy cow but that the amount was very small and the demonstration of its presence depended upon the relative amounts of the serum and milk which were used.

In the same year as the discussion, Kopf(251), working in Schlossmann's laboratory under Bauer, was unable to trace any complement in cow's milk using the same system as Pfaundler and Moro, and myself, except in colostrum, in which complement could be readily demonstrated.

Sassenhagen and Bauer(217) (1909), showed further, that complement appeared in easily demonstrable quantities in mastitis-milk, and that early mastitis could be detected by this test before the fact could be diagnosed clinically.

Sassenhagen(290) (1910) showed that complement is present in considerable amounts in the colostrum, both of cows' and goats, and that it depends for its appearance upon the colostral character of the milk, disappearing gradually although somewhat irregularly, as the milk assumes the normal character.

Moro and Pfaundler(274) failed to obtain evidence of the presence of amboceptor in milk.

In 1908 I believed that I had obtained evidence of the presence of hoemolytic amboceptor in cows' milk. By incubating a mixture of heated ox-serum and guinea-pigs corpuscles a solution (in which, after removal of the corpuscles, I was unable to detect the presence of amboceptor) was obtained and which on being added to milk in the presence of guinea-pig's corpuscles and fresh serum gave hoemolysis. Kopf however states that in his experiments the amboceptor did not become attached to the corpuscles on digestion unless complement were also present. Dr. Bauer, under whom Kopf carried out his work, has since been good enough to show me some figures of his own which bear out his view. Since seeing these figures, this spring, I have not had an opportunity of re-investigating the matter myself.

Both Kopf and Sassenhagen found amboceptor present in colostrum, during the first few days after parturition, hoemolysis occurring with guinea-pigs corpuscles and milk, alone, and the same was found by me in the case of milk from a cow a few days after parturition.

It appears therefore that both amboceptor and complement are present in the early colostrum (the complement persisting until the complete disappearance of the colostral characters), and possibly afterwards in small quantities, although this is a matter upon which authors are not entirely agreed.

Human milk.

Cattaneo(223) (1905) believed that he had obtained evidence of complement in human milk, using human corpuscles. In 1907, however, Frey(232) pointed out that in Cattaneo's experiments there were traces of serum left with the corpuscle which should be removed by washing, and that if this is done no hoemolysis is obtained. Further that Cattaneo's asepsis was not complete, and the experiments were carried out over sufficient length of time(36 hours) to introduce the possibility of bacterial action.

Pfaundler and Moro(271) (1907) were unable to obtain any evidence of the presence of complement in human milk, using the same system as for cows' milk except for the different milk, that is g. pig r.c. ·5 c.c. + inactive ox-serum ·05 c.c. + human milk ·5 c.c. and also rabbits r.c. ·5 c.c. + human inactive serum ·25 + human milk ·5 c.c.

They believed that there was an inhibitory effect when large amounts of milk were used because, if the following mixtures were made and incubated (*a*) 0·5 cc. g. pig r.c. + ·15 human inactive serum + 5 c.c. human milk, and (*b*) 0·5 cc. g. pig r.c. + ·15 human inactive serum + 5 cc. saline and after two hours incubation, 0·5 cc. active human serum was added to both, the result was incomplete hœmolysis in (*a*) and complete hœmolysis in (*b*). They believed this to be due to some alteration of the red corpuscles which rendered them less easily attacked by the hœmolytic substances.

Bauer(215) (1908) showed that the inhibitory effect obtained by Moro and Pfaundler was not due to alteration in the corpuscles, since if the corpuscles were washed after the first incubation, there was no inhibition of hoemolysis. But he believed that there was an inhibitory effect, because if human milk was added to a complete hœmolytic system inhibition of hœmolysis occurred if the milk was added in sufficient amounts. Decreasing amounts of human milk gave increasing amounts of hœmolysis.

In 1908 Pfaundler and Moro(^{276}) showed that the presence of complement in human milk could be demonstrated, if the serum of a rabbit immunised against sheep's corpuscles was used, thus—0·1 cc. 1 per cent. sheep r.c. + 0·1 cc. immune serum (dil) + 0·2 cc. human milk. With this system they obtained hœmolysis, and hence evidence of the presence of complement in human milk.

Noeggerath and Kolff(^{272}) (1909) used a variety of suitable systems, and with the exception of the merest traces on a few occasions, were unable to detect the presence of any complement in human milk. On using the method of Pfaundler and Moro, however, and also other immune sera, they obtained hœmolysis in some cases, and thus demonstrated the presence of complement in human milk.

They did not consider that any real inhibitory effect could be postulated since this only occurs when there is previous incubation of the systems used (cp. Pfaundler and Moro) before the addition of the active serum. By this procedure marked inhibition could be obtained with human milk. If previous incubation of the mixture were not carried out the inhibitory effect could only be demonstrated when comparatively large amounts were used. The same was also found by Moro and Pfaundler.

It appears therefore that complement in human milk can only be demonstrated when an extremely sensitive system is used, which means that it must be present in very small amounts.

Before leaving the question of the hœmolytic factors in milk the work of Bulloch and of Kraus should be mentioned.

Kraus(^{254}) (1901) immunised lactating goats to sheep's corpuscles, and found hœmolytic power in their blood, but the addition of sheep's corpuscles to their milk, did not bring about hœmolysis. Kraus concludes that the immune body did not pass out in the milk (*cp.* Famulener, p. 72).

Bulloch(^{222}) (1902) immunised female rabbits to ox corpuscles, and showed that the milk from these rabbits, which were shortly afterwards in lactation, contained the specific immune body, as was shown by the fact that the addition of complement to the milk, together with ox corpuscles, gave hœmolysis.

Kraus does not appear to have added any complement, which may account for his negative results.

The blood of an actively immune animal is much more sensitive than the blood of one not specifically immune, and this may account for the difference of results obtained between the work upon amboceptor in normal milk, and in that from an immune animal. Normally there is not enough amboceptor present to be detected; it is difficult to believe that traces may not be present.

Summary of Section 1:—

1. Colostrum contains both complement and amboceptor. Amboceptor is only demonstrable during the first few days after parturition, whereas the complement persists longer.

2. The evidence as to the presence of complement in both human and cows' milk after the colostral period is somewhat conflicting. If detected it is found only in quite small quantities.

2.—*The Presence of Bactericidal Substances.*

The factors concerned in bacteriolysis are on parallel lines with those concerned in hœmolysis, the difference being that the amboceptor must be capable of being attached to the bacteria instead of to the red corpuscles, the complement functioning in a similar manner as in hœmolysis.

The amboceptor is specific for different varieties of bacteria, just as it is for different varieties of red corpuscles. Hence in dealing with the bactericidal properties the species of bacteria must be given. In dealing with milk the difficulty is increased owing to the bacteria already present in this fluid, unless extra precautions be taken in milking and later. For this reason some observers have studied the general effect of milk upon the life of the bacterial flora usually present in milk, and others, taking milk freed from the usual flora, have studied the specific action of milk upon different varieties of bacteria.

The importance of ascertaining the viability of bacteria in milk extends much beyond the field of infant feeding, being of great importance in the question of the transmission of disease. In this connection the viability of B. Typhosus and B. Choleræ have been studied especially by the earlier investigators, who were anxious rather to establish the existence or otherwise of the organism than to discover whether there was any decrease in the numbers. Later workers have studied the number of the organisms present in much greater details, with a view to the detection of amboceptors and complement whose presence might perhaps be of value from the biological aspect.

The investigations, as a whole, showed that B. Choleræ died in milk as soon as the milk became sour. (*cp.* Wolffhugel and Riedel(298), Kitasato(253), Heim(243), Uffelmann(294), Friedreich(233), Basenau (213), and others), whilst Weigmann(297) and Hesse(244) both believed that the organisms were destroyed in a few hours by virtue of the inherent bactericidal power of milk. Fokker(234) also obtained an initial decrease.

Schottelius(291) (1896) using B. Diphtheriæ found that raw milk was a much better medium for this organism than either sterilised milk or broth, growth being more rapid in the raw milk.

Conn and Ester(225) (1901) studied the growth of varieties of bacteria at 20° C. The bacterial count was made at the time of the inoculation, and as a rule six hours later. They found that B. Acidi Lactici increased in numbers from the first, although the total numbers of bacteria present remained fairly constant, owing, presumably, to the death of some of the varieties of bacteria for whom milk was an unsuitable medium. Streptococci also tended to show an increase per cent. of the whole bacterial flora present, but there was sometimes a slight absolute initial decrease, during the first six hours. Some organisms which could not be detected in the first estimation, subsequently appeared rather suddenly in the subsequent observations.

Cozzolino(227) (1902) used cows', goats', asses' and human milk. The milk was carefully collected and was then heated on eight successive days to 55-58° C. for one hour. The milk thus treated, was inoculated with B. Coli, and was not found to be a good medium. Of the varieties of milk, human milk showed least growth, an inhibition and even a decrease occurring.

Moro(261) (1901 and 1902) investigated the presence of definite bacteriolytic factors in milk, but was unable to trace the presence of any such bodies in either human or cows' milk.

Klimmer(218) (1903) was unable to detect any bactericidal effect in either asses', cows' or human milk towards B. Coli or B. Typhosus.

Conn and Stocking(224) (1902) did a good deal of work upon the relative numbers of the organisms forming the ordinary bacterial flora of milk. They found that there was nearly always a decrease in the total number of bacteria during the first few hours after milking. They then investigated the changes in the various groups of organisms present, notably those of the acid-forming group. The results could be classified into three groups:—

1. Those in which there was a decrease in the total number of organisms during the first three hours; this was accompanied by a rapid increase in the number of the acid-forming bacteria, not only absolutely, but also in per cent. of the total.
2. A decrease in the total number including the acid-forming ones. This occurred when there were many varieties of acid-forming bacteria present.
3. A slight initial decrease followed by rapid increase, B. Acidi Lactici grew rapidly at first, but afterwards more slowly, decreasing after the first six hours in actual percentage, owing to the still more rapid growth of non-acid-forming varities.

The following table which shows an experiment falling into group 1, gives a good idea of the general trend of the data given.

Time after Milking.	Total Bacteria per c.c.	Acid-forming per c.c.	Liquefying per c.c.	Per cent. of Acid-forming.
Fresh	12,550	1,250	200	10
Three hours ...	12,250	2,000	200	16
Six hours	19,650	2,250	800	23
Nine hours ...	56,900	20,250	550	36
Twelve hours ...	114,250	68,400 (Practically all B.A. Lact.)	1,900	60

P. Sommerfield(273) (1905) put milk through a porcelain filter, and used the sterile serum thus obtained to investigate the bactericidal properties of the milk. He found no bactericidal power towards B. Coli or B. Typhosus.

Kolle(250) (1905) using fairly germ-free milk obtained some evidence of the inhibtion of growth of B. Choleræ but not of B. Coli or of B. Typhosus.

Koning(252) (1905) carried out experiments upon the numbers of bacteria present in the same sample of milk directly after milking and later. He found as other observers had done, that there was an initial decrease in the total number of bacteria. This decrease was most marked in milk which had been collected as far as possible free from bacterial contamination, being less marked in dirty milk. The optimum temperature was 37° C. The phenomenon was absent in milk which had been boiled, but was retained for a longer period if the milk was kept cool. Koning believed that milk contained substances inhibitory to the growth of bacteria, which were probably derived from the blood.

Rullman and Trommsdorff(258) (1906) also found that the inhibitory effect upon the ordinary bacterial flora was increased in carefully-collected milk, and lasted almost throughout the first day, even at room temperature. This property, together with the leucocyte count was increased in mastitis.

St. John and Pennington(251) (1907) found that bacteria grew more readily in pasteurised milk than in raw milk, and that the acid-forming organisms in particular grew much more rapidly.

Moro(261) (1907) re-investigated the action of raw cows' milk upon typhoid bacilli, and found that raw milk had a slight action, but that this was absent in boiled milk or in the serum obtained by putting milk through a porcelain filter. The inhibition was not great, nor was the growth in the boiled samples always large. The inhibitory effect was almost entirely destroyed by heating to 56° C. for half-an-hour.

The following figures illustrate the results obtained by Moro.

—	Hours after Milking.			
	0.	1.	3.	7.
Bacteria per c.c. in raw milk	466	357	258	4,000
Bacteria per c.c. in boiled milk	222	285	1,960	Infinite.

Moro discusses the literature and points out that the different results obtained by the various observers, means that there is no marked action, owing to the small amount of the bactericidal substance present.

Myer Coplans(226) (1907) inoculated raw milk and boiled milk (heated to 100° C. for one hour on two successive days) with cultures of B. Coli. He obtained slight bacteriolysis and inhibition of growth for six hours after milking at 20° C., and partial inhibition for some hours more. At blood-heat the same inhibition only lasted for one hour, and the partial inhibition for from 2-6 hours after milking. At 0° C. the inhibition lasted for 24 hours. The rate of growth in boiled milk was taken as the standard of growth without inhibition. He points out that infants fed upon shop milk can obtain little benefit from this power of milk.

Rosenau and McCoy(283) (1908) studied the action of milk on bacteria. They showed that the apparent bactericidal effect which occurred in fresh milk was really due to agglutination of the bacteria, and could be shown to be entirely absent if the agglutinated masses of bacteria were broken up before plating out.

This decrease occurred only in the first 6-8 hours after milking, and was not found in boiled milk or in milk heated to 80° C. It was not effected by freezing, but was hindered by dilution. The action was specific, since it was not the same for different species of bacteria, nor was it the same in different milks, and even in different samples from the same animal. They also showed that the agglutinating and therefore the inhibitory effect could be apparently restored by adding a drop of typhoid agglutinating serum, as shown in the table below.

They also showed that the action was not due to leucocytes, since it was present in milk which had had the corpuscles removed by centrifuging. The breaking up of the clumps was accomplished by drawing the milk up and down a fine pipette pressed against the bottom of the vessel.

The milk was almost germ-free, and the samples after inoculation were kept at 37° C.

—	BACTERIA PER LOOPFUL.					
	At once.	2½ Hours.	4½ Hours.	6½ Hours.	8½ Hours.	
					moderate shaking.	mixed with pipette.
B. Typhosus raw milk ...	1,880	1,380	1,060	1,480	1,980	12,200
Do. boiled milk ...	2,120	4,020	*a. b.* 800,000	*a.*	*a.*	*a.*
Do onedrop typhoid serum added.	2,100	2,040	1,920	2,300	1,260	*a. b.* 20,000
Do. boiled	2,280	2,360	7,020	6,480	10,860	*a. b.* 60,000
B. Typhosus in broth, serum added.	1,830	970	2,920	9,180	11,160	*a. b.* 100,000
Organisms in milk alone ...	0	1	2	2	2	46

a. = Innumerable. *b.* = About.

This table is most interesting and shows the effect due to agglutination.

Much(269) (1908) showed that milk treated with hydrogen peroxide retains its apparent bactericidal properties much longer than ordinary milk. If however the hydrogen peroxide be added as late 20 hours after milking, then it has no influence, the normal apparently inhibitory effect having worn off before the addition of the disinfectant.

Bartelli(212) (1909) believed that the decrease in the ordinary bacterial flora was due to the gradual development of acidity in the milk owing to the growth of lactic acid organisms. If the milk was kept cool during the period of usual inhibition then the acidity did not rise and there was no inhibition.

In the following table he showed that the inhibition starts when the milk is kept at the optimum temperature for the development of the lactic acid organisms, namely 37° C.

Time after inoculation.	Milk I (kept at 0° C.).		Milk II (at 0° C. for 6 hours, then at 37° C.).		Milk III (at 37° C.).	
	Bact. per c.c.	Lactic acid per cent.	Bact. per c.c.	Lactic acid per cent.	Bact. per c.c.	Lactic acid per cent.
At once ...	143,000	0·0011	143,000	0·0011	143,000	0·0011
Three hours ...	145,000	0·0015	145,000	0·0015	140,300	0·0133
Six hours ...	145,600	0·0016	145,600	0·0016	129,000	0·1226
Nine hours ...	149,000	0·0018	141,250	0·0106	190,000	1·0037
Twelve hours .	151,000	0·0025	127,000	0·0762	253,000	1·1009

The inhibition shown by these figures cannot be considered to be other than very small.

Sassenhagen(290) (1910) working with colostrum obtained evidence of bactericidal effect upon B. Coli, which was much stronger than in ordinary milk.

Bub(221) (1910) carried out numerous experiments upon the action of colostrum upon various organisms. He first of all tested the effect upon the ordinary flora of milk collected without any special precautions. He obtained evidence of some inhibition at 15-18° C. and less at 37° C., viz.:—

Time after Milking.	Bacteria per c.c. at 15–18° C.	Bacteria per c.c. at 37° C.
At once	783	848
Two hours	604	754
Four hours	716	940
Six hours	818	5,800
Eight hours	739	26,340
Ten hours	636	70,000
Twelve hours	1003	150,000

Bub then took milk collected with all possible precautions and inoculated it with various organisms, B. Coli, B. Paratyphosus, and B. Pyocyaneus, and obtained a well marked decrease in the bacterial count of the samples inoculated with each of the organisms, the effect wearing off in a shorter time at 37° C. than at 15-18° C., viz.:—

Time after inoculation.	Count of B. Coli.		Count of B. Paratyp.		Count of B. Pyocyaneus.	
	At 15–18° C.	At 37° C.	At 15–18° C.	At 37° C.	At 15–18° C.	At 37° C.
At once ...	3,345	3,648	3,582	3,453	13,425	12,785
In two hours	1,372	711	—	2,952	11,520	13,644
In four hours..	887	2,160	3,489	2,840	7,485	18,870
In six hours...	872	27,000	—	8,570	6,892	50,000
In eight hours	845	70,000	3,172	19,470	22,680	Infinite.

If the tubes were well shaken before taking the bacterial count no inhibition was detected although the count is much higher in boiled milk than in raw.

—	No. at once after inoculation.	No. after 6 Hours. (Moderate shaking.)	No. after 6 Hours. (Strong shaking.)
B. Coli in raw milk ...	1,184	8,120	49,000
Do. sterilised... ...	1,145	2,000,000	—
B. Parat. in raw milk ...	6,342	3,873	57,000
Do. sterilised ...	6,480	Infinite.	—
B. Pyocyaneus in raw milk	11,870	17,760	Infinite.
Do. sterilised	15,084	Infinite.	—

There seems therefore little doubt as to the important part played by agglutination. The further fact that the growth is less rapid in raw milk than in boiled, does not necessarily postulate any bactericidal or inhibitory effect as compared with the rate of growth in sterilised milk, because as has been pointed out by various authors, this may merely be due to the fact that the chemical changes which take place in prolonged boiling or sterilising may render the milk a better medium, and growth proceeds at a more rapid rate.

Gutzeit(237) (1911) studied the bacterial count in filtered milk, and did not think that the count increased any more rapidly in the filtered milk than in the control milk; he points out that allowance must be made for the length of time which elapses in the process of filtering, the temperature, &c.

Kleinschmidt(217) (1911) examined both human and cows' milk for the presence of bacteriolytic amboceptor for B. Coli and B. Typhosus. He believed that he had obtained evidence of small amounts of these bodies in both forms of milk. He took carefully collected milk which was almost germ-free. This was inoculated with cultures of B. Coli and B. Typhosus, the amount of the inoculation being determined by plating out. He endeavoured to avoid agglutination effects by shaking, but details are not given. Kleinschmidt concludes from his figures that amboceptor for both Coli and Typhosus are present in cows' milk. In the colostrum of a cow two days post-partum he found much stronger effect, and considers that complement is present as well. The figures given below are typical of the results obtained in various experiments.

Number of bacilli inoculated per cc. were B. Typhosus 1,720, and B. Coli 3,000.

—	Typhoid after 3 hours.	Coli after 3 hours.	Coli after 7 hours.
Raw milk	128	2,800	30,000
Milk heated to 56° C. ...	2,840	6,480	Infinite.
Do. + ·01 c.c. human serum	108	364	50,000
Milk heated to 100° C. + ·01 human serum.	10,000	50,000	Infinite.

He thinks that bacteria probably grow quicker in boiled milk than in raw, apart from the possible destruction of bactericidal substances.

The work upon the bactericidal substances in *human* milk must now be considered; it is neither large nor very definite.

Honigmann(215) (1893) inoculated human milk with B. Typhosus, B. Coli, and Staphylococcus Aureus. He obtained some inhibition of growth but no bactericidal power within the first 6½ hours after milking. The inhibition he believed to be due to the protein, since if blood was diluted until the percentage of protein was that of the milk only an occasional decrease was noticed, and frequently no action at all.

Moro(261) (1901) failed to obtain any trace of bactericidal substance in human milk, whilst Cozzolino(227) (1902) found that there was an initial inhibition of growth, and even a slight decrease in number.

Klimmer(248) (1903) was able to demonstrate that in human milk there is occasionally a slight inhibitory effect upon B. Coli and B. Typhosus in the first few hours.

Moro(264) in 1907 believed that he had obtained evidence of traces of bactericidal action upon typhoid bacilli, but Noeggerath and Kolff(272) (1909) criticised his results as not really showing any effect and they themselves were unable to demonstrate any. They found, as did Moro, that boiled milk, and milk put through a Berkefield filter did not give any inhibition, and agreed with Moro that boiled milk probably provides a better medium for growth than raw milk. It is not necessary to enter into a detailed discussion of the results obtained by these observers. Probably traces are present but it may be that the amount is frequently too small to be demonstrable, and varies in the different samples of milk.

Kleinschmidt(247) (1911) using the same procedure as for cows' milk worked with human milk and human colostrum, and believed that he had obtained evidence of the presence of amboceptor for both B. Coli and B. Typhosus, and thinks that it may be of use to the infant if it gets into the blood (*cp.* below, p. 69 *et seq.*).

He gives the following figures:—

—	B. Coli. Inoculation 3,600 per c.c.		B. Typhosus. Inoculation 2,320 per c.c.			
			Milk.		Colostrum.	
	After 3 hours.	After 7 hours.	After 3 hours.	After 7 hours.	After 3 hours.	After 7 hours.
Raw milk approx. ...	30,000	Infinite.	5,630	10,000	3,840	10,000
Heated to 56° C. ...	50,000	Do.	5,800	20,000	5,600	10,000
Do. + ·01 human serum.	3,360	20,000	44	1,040	1,160	4,400
100° C. + ·01 human serum	50,000	Infinite.	5,780	Infinite.	6,340	Infinite.

If it be admitted that boiled milk is a better medium for the growth of these organisms, then the figures do not appear very conclusive.

3.—*The presence of precipitins.*

When a foreign protein gains entrance into the blood-stream of any animal, it evokes a reaction in the tissues of the animal, a substance being produced which is capable of forming a precipitate with that foreign protein and thus throwing it out of action.

Such bodies which are capable of throwing out foreign proteins are known as precipitins. The reaction is an exceedingly delicate one, and very small amounts of foreign protein can be detected.

This reaction has, as will be seen later, been largely used in connection with the absorption of protein from the alimentary canal.

Precipitins are not normally present in serum, so that unless under exceptional circumstances they are not to be expected in the milk. Langer(257) (1907) however showed that the antigens or substances which can produce precipitins in the blood of another species pass out into the colostrum in considerable amount, and that cows' colostrum has a much higher content of these substances than later milk.

4.—*The presence of agglutinins in milk.*

It has already been shown that milk has agglutinating power in dealing with the bactericidal action of this fluid, and it will not be necessary to dwell much longer upon this point. Kraus(254) (1901) found that artificial agglutinins if present in the blood were passed out into the milk and Langer(257) (1907) showed that the agglutinating power of colostrum is greater than that of the later milk. This has also been shown repeatedly in the case of women who had suffered from typhoid shortly before parturition (*cp.* Bauer(216) who gives a full account of the litreature).

5.—*The presence of anti-toxins.*

There is abundant evidence to show that anti-toxins if present in the blood of the mother pass out into the milk. This was first shown by Ehrlich and Wassermann(229) (1894) who found that if diphtheria anti-toxin were present in the blood of a lactating animal it passed out in the milk in considerable amounts, about one-fifteenth to one-thirtieth that of the blood. If the anti-toxin content of the blood were increased the amount present in the milk also rose.

Most of the work upon the presence of anti-toxins has been carried out in connection with the absorption of these bodies and the production of passive immunity, and will be considered under that heading (*see* p. 70 *et seq.*).

Summarising the results given in Division I. it appears that:—

1. Colostrum contains substances (*a*) acting hœmolytically upon certain blood corpuscles, and (*b*) inhibiting the growth of bacteria, whether through bacteriolysis or agglutination.
2. Later milk may contain traces of these substances but if present at all they are present in very small amounts.

3. The apparent bacteriolytic effect possessed by raw milk seems to be due to its agglutinating powers; this power is lost in the early hours after milking.
4. If specific immune substances are present in the blood of the mother they pass out in the milk but are present in smaller amounts than in the blood.

DIVISION B.—ON THE ABSORPTION OF THE SO-CALLED "PROTECTIVE SUBSTANCES" IN MILK.

It has been shown in the preceding division that traces of the bodies concerned in the production of hœmolysis and bacteriolysis may be present in both human and cows' milk, and that agglutinating power is found in fresh milk. Those observers who have worked with colostrum, are all agreed that these powers are greater in colostrum than in the later milk.

If these substances are to be of any value to the infant in producing immunity to disease they must enter the blood stream. The general experience is that these actions are due to some group or body, attached to the proteins of the blood. If therefore these substances are to reach the blood-stream either the protein to which they are attached must be absorbed as such, or the bodies must be able to withstand the action of the digestive juices upon the proteins. In either case the question as to the origin of the protein has to be considered, that is, whether it is native or foreign protein.

The really important question for consideration in the present report is whether the substances are absorbed, the action of the digestive juices being relatively a subsidiary one, since if the substances are not absorbed they cannot be of value to the organism in their specific capacity.

Observers are by no means agreed as to the action of the digestive juices upon these bodies, and this point cannot be looked upon as settled.

Michaelis and Oppenheimer(260) (1902) found that the protective bodies were destroyed by digestion owing to the breaking down of the proteins to which they were attached. Obermeyer and Pick(279) (1902) did not however agree with this view, and Sacconaghi(286) (1904) obtained the production of a precipitin by injecting a solution of protein which had been digested until it no longer gave the protein reactions.

Schlossmann and Moro(292) (1904) showed that human and cows' protein were quite distinct bodies producing different precipitins on injection, and they concluded that native protein could be absorbed as such from the alimentary canal of the infant, and carry with it the protective properties, whereas foreign protein was broken down in the process of digestion.

Bauer(214) (1905) reviewing the situation, came to the conclusion that the action of the digestive juices upon these bodies, and the question of the absorption of protein was still undecided. In 1908, however, he stated (at the Meeting of German pediatricians which took place at Köln, and also private information) that complement if put into an infant's stomach in large amounts, is destroyed, no complement being detected if the fluid is withdrawn five minutes afterwards.

The *absorption* of the protective substances and of protein must now be considered.

The absorption of the bodies concerned in immunity has been studied by many observers, the investigations being for the most part concerned with the production of passive immunity in the suckling animal. Mothers of different species have been injected with anti-toxins both before and after parturition and the blood of the suckling examined for the presence of the anti-toxin, which was injected into the mother. The absorption of protein has been investigated by testing the blood of the young animal by the precipitin reaction.

Absorption of complement and amboceptor have also been sought for.

A few experiments have been carried out on mothers who have been made actively immune to certain bodies, and these will be dealt with separately. As a whole there is considerable evidence to show that in the first days of life protein and its attached properties may be absorbed as such, and thus produce a passive immunity; the length of time during which this capacity lasts after birth depends upon the species of animal.

Later, after the early days of life, absorption of protein from the intestine only occurs in cases of disease or other disturbance of the intestine, or in the presence of excess of protein. This last, however, is probably dependant upon the former factors, since it is a well-known fact that excess of food-material will give rise to intestinal disturbances.

As the greatest amount of work has been carried out in connection with the absorption of *anti-toxins* this will be considered first.

Ehrlich(228) (1892) showed that temporary immunity to abrin, ricin, robin and tetanus, can be conferred by suckling, upon the young of a mother who is herself immune to these substances. He found that this immunity lasted well, for about a month after birth in the animals (mice) used by him; at the beginning of the third month of life it was much reduced, being absent at the end of that month.

Romer(280) (1901) injected diphtheria anti-toxin into a pregnant mare shortly before parturition. The anti-toxin content of the blood rose, and after parturition anti-toxin appeared in the milk, in a strength of about one-tenth that of the blood. The foal's blood at time of birth contained no anti-toxin, but after birth it soon appeared and the amount present continued to rise until the 12th day, when it gradually sank. Further injections into the mother gave rise of anti-toxin in the milk, but the anti-toxin content of the foal's blood fell persistently.

Hamburger (1907) immunised female mice to ricin; after parturition one of the immunised mothers suckled the young mice from a normal mother, whilst another immunised mother suckled her own young. Both families developed a high immunity of about the same degree.

Further, a normal kid was fed by a mother who had been injected with tetanus anti-toxin. The experiment was commenced when the kid was 12 days old, and was continued up to the 24th day; only a small amount of absorption took place. Hamburger concludes that only a part of the protein is absorbed whether of the same species or not.

Much and Römer(267) (1907) obtained evidence of the absorption of tetanus anti-toxin in the early days of life. They believed that the degree of absorption was greater if the anti-toxin were derived from the blood of the same species than if it were derived from that of a foreign species. A calf was fed from birth with the milk of a mother who had received tetanus anti-toxin: the amount of anti-toxin in the milk was estimated, as was also that in the calf's serum. It was found that about one-twentieth of the anti-toxin present in the milk was absorbed. If, however, the anti-toxin (derived from a horse) were mixed with the milk from a normal mother and fed to the calf, (the same amount of anti-toxin being added as was found to be present in the milk), an absorption of only 1/200 of the amount occurs.

Also if the calf is not allowed to suckle from the immunised mother until the 5th day after birth, only about 1/2000 is absorbed.

Working with Happich, Much(268) found practically the same for the human infant.

Tetanus anti-toxin was injected into a woman (1) on the day before the confinement, and (2) four days after this event. At birth the infant showed no anti-toxin in the blood, but after four days it had absorbed about one-fourth of the total amount calculated to have been present in the milk, taken from the breast. Between four and eight days of age the infant absorbed one-twelfth of the total anti-toxin present in the milk. If the milk were drawn off and the corresponding amount of anti-toxin (from a horse) added to it, then only about 1/64 was absorbed from the 2-6th days of life, and 1/192 from the 4-8th days.

Much and his collaborators, suggest that the difference is due to a change in the attachment of the anti-toxin, which is taken in by the mother attached to horse protein, and is passed over (as it were) to the human protein in the course of its passage through the human organism, and is thus turned out in the milk as human protein, and is more readily absorbed. Evidence of the presence of horse protein in the milk of the mother could not be obtained by the precipitin reaction, nor by deviation of complement. Further if the human milk containing the anti-toxin was injected into a rabbit, neither human nor horse protein could be found in the milk of that rabbit.

Hamburger(241) (1907) wrote disagreeing with Much and his fellow-workers. He found that if lactating rabbits are injected with tetanus anti-toxin after parturition this appears in the milk about 26 hours after birth. It appeared as horse anti-toxin, and this could be demonstrated by the ordinary precipitin test; it also appeared in the blood of the young animal, in about 1/400 the strength of that in the milk, but its presence could only be detected by the usual method of injection of the serum into mice. Fifteen days after parturition the anti-toxin had completely disappeared from the mother's blood, from the milk, and from the blood of the suckling rabbit.

Römer(281) (1909) carried out further work in this direction, being stimulated thereto by Salge's work upon the absorption of immune body and agglutins (*see* p. 74). He points out that Salge was not using the protein of the same species, and considers this of great importance. He used horse serum and a foal for the purpose of his experiments. The foal was 25 days old when the work was started, and the mother was then injected with tetanus anti-toxin, of known strength and amount. The amount passed out in the milk was estimated on the 4th, 6th, and 8th days after injection. It was calculated that the foal took 15 litres per diem, and working upon this basis it was found to have absorbed one-eighteenth of the total amount of anti-toxin which was present in the milk. Calculating from data obtained upon another horse he found that if the same amount per kilo of body-weight of anti-toxin had been injected into the foal instead of being absorbed from the intestine, the blood would have contained approximately 12 times as much anti-toxin; that is to say the amount of absorption which took place by the intestine was about one-twelfth of what it would have been had the same amount been injected.

The same foal then received milk to which anti-toxin had been added, and no rise of anti-toxin content occurred in the foal's serum, which was now practically free from anti-toxin. He then injected the mother with another dose of anti-toxin and obtained anti-toxin in the milk, but the foal's blood only showed a very slight rise of anti-toxin amounting to 1/10,000 of an anti-toxin unit per cc.

Working with Sames, Römer(282) (1910) injected a pregnant sheep with tetanus anti-toxin on the 2nd and 6th days before parturition. The blood of the lambs which had suckled from the mother who had been injected, showed anti-toxin up to about 4-6 months of age. Taking his previous results (*cp.* also p. 70) as showing that anti-toxin is not absorbed after the first few days of life these results showed that it is very slowly excreted.

Vaillard(296) (1896) used mothers which were actively immunised to bacteria, and found that little or no immunity was obtained by suckling. He immunised guinea-pigs to B. Cholera and B. Tetanus, and rabbits to B. Anthracis.

Famulener(231) (1912) also worked with actively immunised mothers. He immunised pregnant goats to sheep's red corpuscles, and showed that the offspring after suckling had acquired a passive immunity to sheep's corpuscles, the anti-bodies being found in the colostrum. The content of these bodies in the colostrum was often higher than that of the serum of the mother. If the immunisation of the mother was commenced after parturition, no immunity was conferred upon the young animals by suckling, although anti-bodies could be detected in the milk, but in smaller amounts than in the colostrum. Absorption of the anti-bodies only took place in the early days after birth.

He also found that after the colostral period a high degree of immunity in the mother is required before the anti-bodies pass out in the milk.

These interesting results are in harmony with those already mentioned.

There is a fair amount of evidence dealing with the absorption of protein in the days of life after the early ones. As a whole absorption does not normally appear to take place.

Römer(280) (1901) found no absorption of anti-toxin from the alimentary canal of adult sheep and guinea-pigs, and believed that in the sheep the anti-toxin was bound by the fæces.

Salge(288) (1904) fed babies upon human milk to which diphtheria anti-toxin had been added; in a child four days old when the experiment was started no absorption could be detected, even after 20 days: in another child 34 days old and fed for 21 days a negative result was also obtained.

McClintock and King(258) (1906 and 1909) were able to obtain some degree of passive immunity in children by the absorption of diphtheria anti-toxin from the alimentary canal. In order for this to take place it was found necessary to prepare the intestine and stomach by previous fasting, and by the administration of morphia. Further the anti-toxin had to be given with certain precautions. Unless these precautions were observed there was no absorption of anti-toxin, or so little that it could be considered negligible.

The *absorption of protein* has also been studied, and the protein tested for in the blood by means of the *precipitin* reaction.

Hamburger and Sperk(240) (1904) failed to obtain absorption of protein in a calf of three days old, or in infants of from 5-13 weeks old, to whom horse serum was given.

Ganghofner and Langer(235) (1904) found that egg-white was absorbed by young animals during the first few days of life, viz.:—

In puppies it occurred up to the 6th day.
In kittens it occurred up to the 8th day.
In rabbits it occurred up to the 7th day.

In goats absorption was already negative at the 8th day but was found to be positive in one new born kid. In infants they considered that the absorption might last three weeks. It, however, large doses were given absorption could be obtained in older animals. The passage of these substances calls up the appropriate reaction of anti-body formation, which occurs to a fair degree.

Moro(266) (1906) examined the blood of artificially-fed and diseased infants, by the precipitin reaction. The blood of an atrophic, overfed infant, presumably suffering from some degree of intestinal derangement, gave an intense reaction. He suggests that this may be due to functional derangement of the intestine due to the feeding with excess of foreign protein. Later he examined 22 more cases of atrophic or otherwise diseased children, and obtained positive results in only two cases. Using the method of complement deviation, in seven cases examined four were positive.

Langer(257) (1907) showed that colostrum was much richer in antigens than was later milk, and that the blood of young suckling animals in the first days after birth was rich in the same antigens as were found in the milk, these being the same as in the mother's blood. The serum of calves at birth gave no trace of precipitin reaction, but this could be obtained from 6-8 hours after the first feed of colostrum, the reaction increasing up to the second day, and then falling to a lower level. Later the level again rises.

Dealing with adult rabbits Uhlenhuth(295) (1900) showed that some absorption of egg-white will take place in adult rabbits if the amount given be very large. The reaction was quite definite, but it could not be got to increase.

Mayerhofer and Pribram(259) (1910) took pieces of the small intestine of quite young guinea-pigs, goats, and rabbits, and also took similar pieces of the intestines from animals of the same age and species, whose intestines had been made abnormal by feeding them upon raw cows' milk. Suitable precautions being taken, the loops of intestine were filled with rennet, tetanus anti-toxin, egg-white, &c., and suspended in saline solution, the amounts of these substances found outside the intestinal mucous membrane being estimated. They found that the permeability of the abnormal intestine was greatly increased.

Van Alstyne and Grant(211) (1911) made Thierry-Vella fistulae in dogs, and after the fistula was healed the dog was anæsthetised, and egg-white injected into the fistula; the amount of egg-white given is not stated. They obtained evidence of some absorption of the protein because the blood coming away from the loop contained sufficient to produce hypersensitivity in a guinea-pig into which it was injected.

Both Mayerhofer and Pribram's, and Van Alstyne and Grant's experiments are open to the doubt as to the condition of the intestine. It is unlikely that an intestine suspended in saline is in the same condition as an intestine *in situ*, and in the possession of its normal blood-supply. Also in the case of the fistula the condition of the mucous membrane of the fistula is very probably modified, and there is in addition the effect of the anæsthetic.

The evidence upon the *absorption of agglutinins* is not large. Kraus(256) (1901) found no absorption of typhoid agglutinin in young rabbits whose age is not given, but which appear from the context to have been about three weeks old.

Bertarelli(219) (1905) induced passive immunity in young animals during the first few days of life, but he did not consider that it was very striking.

Salge(289) (1907) fed a child nine weeks old, upon the milk of a goat which was immune to typhoid. He obtained no evidence of the absorption of any agglutinin from the intestine of the infant. He concludes that there is no absorption of foreign protein in the infant after four days of age (*c.p.* his other experiments, p. 73), and he did not feel justified in trying the effect of foreign protein upon a younger infant.

It appears to be well-established that absorption of protein does not take place directly from the alimentary canal after the first few days of life.

Before summarising the whole question, it will be of interest to consider the work which has been carried out in connection with the content of the infants blood at birth as to immune substances, and the gradual development of these substances.

Halban(238) (1900) took blood from the unbilical cord, and from the mother. He found that the infants blood was much poorer both in agglutinating power and in hæmolytic power, than that of the mother.

Moro(261) (1901) investigated the complement content of infants blood, in relation to the method of feeding. He found that the bactericidal power of breast-fed children was higher than that of artificially-fed babies, and that this was not merely a question of health because weakly breast-fed babies often had a higher content than healthy artificially-fed ones. Moro states that if the breast-fed baby was put on cows' milk that the bactericidal power fell almost at once. Moro at that time was unable to find any complement in cows' milk, and in human milk. Believing the bactericidal power to be due to direct absorption he was led to the conclusion that complement must be present in the form of a " gen " or alexogenic substance.

Halban and Landsteiner(239) (1902) showed that the blood of infants at birth is deficient in amboceptor, and likewise in almost all the bodies which produce immunity.

Bertarelli(219) (1905) tested the power of young animals to form bactericidal and hæmolytic substances by absorption from the intestine. He fed new-born puppies and rabbits on dead cultures of typhoid bacilli and on foreign red corpuscles. There appeared to be no power of forming active immune substances in these young animals during the first four or five days of life. The power then commences, and at the 15-20th day of life these substances may be present in fair amount, not so much inferior to that of the adult. Rather older animals were also fed upon typhoid bacilli, and most of them became very ill, many dying, but agglutinin was formed in the blood. Presumably the bacilli and the red corpuscles gained entrance to the system owing to the derangement of the alimentary canal.

V. Behring(218) (1903) states that tubercle bacilli can pass the mucous membrane of the young animal in the first few days of life, and later only when large doses are given. This agrees with Bertarelli's work.

Since this date a considerable amount of work has been carried out upon the relationship of the complement content of the blood of infants to their food. The greater part of the work has been carried out by the Munich School of Pediatricians under the guidance of Pfaundler and Moro.

Pfaundler(273) (speaking on behalf of his school in 1907) said that the blood of 83 infants had been examined, and it had been found that the blood of the infant at birth contains no amboceptor, but that the complement is nearly as powerful as in the adult. Immediately after birth the complement content falls and its subsequent rise depends somewhat upon the character of the feeding. If breast-fed the content rises, and if artificially-fed it may fall at first, but may rise again afterwards. To some extent the complement varies with the condition of the child, but this is not absolute. Injection of saline may increase the complement content. Later (1908) Pfaundler(277) and Moro and Potpeschnig(265) showed that as a whole the healthy infants have a higher complement content than the unhealthy ones, but that in cases of fever, or of the severe condition described by Finkelstein as " Intoxikation " the complement content of the blood is high; should such a condition show a low content the prognosis is very bad. He found that amboceptor is not present in the blood of infants at birth but that the amount rises after birth. In pigs and cows, however, there is hardly any development of amboceptor during the first year. Pfaundler quotes the case of a new-born kid, whose complement content rose during the first few days when it was fed on milk from the breast, but afterwards when fed upon boiled mothers milk the content fell, and the animal very soon developed an attack of constipation and other troubles. On restoring the animal to the breast, the troubles passed away gradually, and at the same time the complement content rose. The weight curve rose steadily the whole time even during the constipation. Bauer pointed out at the time that the results were really due to the sickly condition of the animal, a fall in complement was what occurred in sickly animals, and that this explained the results.

Koch (1909) also found that the complement content of the blood rose in fever; as a whole it tended to increase with improved body-weight and to fall with loss of the same, that is, it depends upon the condition of the cells of the body. In the cases he investigated there was one of a child which was weaned suddenly, and lost complement content; another of a badly nourished artificially-fed child in whom the content improved when put on the breast. He found that the complement content was much less in the blood of the embryo than in the mother, and that amboceptor was not present in the blood of new-born animal, the complement content varying very much.

Kaumheimer(216) (1909) supplementing the numbers of children worked upon by Moro, came to much the same conclusions as those already given by Pfaundler for his school. As a rule healthy children had high complement content and sickly ones a low one, but there was no absolute rule, and it was impossible to dogmatise upon the matter. Injection of saline raises the hœmolytic power but is is not clear whether this is due to rise of amboceptor or complement.

Gewin(236) (1909) in Schlossmann's laboratory, investigated the amboceptor-content of new born children for sheep's blood. In breast-fed children this only appears in exceptional cases under six months of age, but in artificially-fed ones it usually occurs earlier. (Amboceptor for sheep's blood is normally present in human blood.) Gewin thinks that the earlier formation of this amboceptor in the blood of artificially-fed children is due to infection, which induces the formation of amboceptor. The absence of amboceptor in the blood of infants at birth had also been found by Bauer, who stated this fact in the discussion on Pfaundler's paper given at Köln in the previous year. Bauer also pointed out the difficulty of investigating the complement content in infants, owing to the fact that immune sera could not be used. He believed the complement content to depend upon the health of the animal.

Findlay, Foa, and Noegerrath(271) (1909) estimated the complement content of 98 infants. They found that some did show increase and others did not do so, as their condition improved. One child who was breast-fed and not doing well, with no detectable complement at all, improved at once on artificial feeding. The content increases with the age as does also the amboceptor content. They conclude that the complement content is not a satisfactory basis to use as a foundation for any physiological argument.

Summary of the results of Division B :—

From the evidence given in Division B it appears that—

1. Absorption of protein and hence of the attached immune substances takes place directly during the first few days after birth.
2. Foreign protein is not absorbed as such except in the early days after birth.
3. It is possible that native proteins may be absorbed directly for a longer period.
4. Protein can apparently be absorbed at a later age in significant quantities in cases of disturbance of the alimentary canal, or of presence of excess of protein.

PART IV.—GENERAL SUMMARY AND CONCLUSIONS.

The work described in Part II. shows that most of the ferments, concerning whose presence in milk so much has been heard in the last ten years (*cp.* Escherich) are derived from the bacteria which are found in milk.

There is no evidence to show that uncontaminated milk contains any ferments capable of assisting in the digestion of food by any of the processes of digestion at present known to us.

The only ferments present in uncontaminated milk are those which are well-known to be present in large quantities in the blood, and ferments (whose presence is not firmly established) which act upon substances not known to occur in the processes of digestion.

In milk, the content of ferments which are similar to those which occur in the blood is found to be increased in quantity at periods when the mammary gland is not in a condition of good working activity; such as at the beginning and end of lactation, in mastitis, and in the case of poorly-acting glands.

It is, I believe, universally conceded that milk from a gland in these conditions contains exudate from the blood; the healthier the gland the less the exudate. It follows, therefore, that the traces of such ferments as are found in the milk under normal conditions, are present because they have passed out from the blood either by filtration or exudation. A precisely similar explanation holds good for the presence in milk of substances concerned in the production of immunity. Here also, it has been shown that these bodies which are normally present in the blood are present in increased amounts in the milk at precisely the same periods as those of its increased ferment content, namely, during the production of colostrum or during mastitis; and that when the mammary gland is in full working order little or none of these bodies appears in the milk. Also if the blood passing to the gland contains specific immune substances these substances appear in small quantities in the milk.

The mammary gland is not only a secretory organ but also an organ admitting of the passage by filtration or exudation, of traces of the substances which are brought to it in the blood-stream.

The value of these materials to the infant evidently depends upon the degree of absorption which takes place from its alimentary canal. From the work quoted in Part II. it appears that absorption of protein and hence of the immune substances which are attached to the protein molecule, may take place directly during the first few days of life. This capacity is however of very short duration, especially for foreign protein, which must be broken down before it can be absorbed.

The oft-repeated assertion of the value to the infant of raw cows' milk fades away when the facts are examined, since, in cows' milk it is found that these so-called "biological substances" are not absorbed in the alimentary canal but are destroyed there.

These considerations also explain the results obtained by those who have investigated the comparative nutritive properties of the raw and boiled milk of a foreign species; these results were fully summarised by me (255) in a recent report to the Local Government Board, and it was shown that in dealing with the milk of a foreign species, boiled milk gave perhaps slightly better results than raw milk. In dealing with the milk of a foreign species the real question at issue is that of the chemical changes which take place on heating. The Berlin figures worked up by me, and general clinical experience afford conclusive evidence that raising milk to the temperature of boiling water does not destroy or apparently injure any of the chemical substances necessary for the health of the child. Above this temperature and even at this temperature for any length of time, chemical changes are known to occur. (In this connection the Addendum to this report may be referred to.)

If the milk of the same species be now considered, a somewhat different aspect is put upon the whole question, because there appears to be some degree of evidence that native protein is absorbed as such, for a longer period than foreign protein. The transitory nature of immunity procured by suckling does not however lead to the supposition that this occurs to any extent, and the evidence all goes to show that the amount of protein which is absorbed as such at any time forms only a small part of the total amount present.

The question finally resolves itself into the chemical value of the food material, and it is this fact which brings about the difference commonly observed between the condition of the average breast-fed baby and the average artificially-fed infant. The substances in the mother's milk are more easily assimilated than those of a foreign species, especially in the early weeks of life, when the organs of the infant are gradually getting into working order. Considering for a moment the almost certain absorption of protein during the first few days of life it would appear to be very important that the organs should receive native protein. Without entering upon the much discussed question of the effect on the infant of the introduction into the blood stream soon after birth of foreign protein, the mere fact that precipitins are formed by the organism upon the introduction of foreign protein for the express purpose of throwing these substances out of action, would of itself seem to indicate that their presence is not desirable. It has been stated that the injection of foreign protein calls forth less resistance in quite young animals than in older ones, but this probably does not mean that such substances are harmless, but that the organs are not yet sufficiently mature to be able to form precipitins. It has been shown by Schlossman and Moro that the proteins of human milk and of human blood are biologically identical, hence the absorption of native protein brings about no disturbance to the young organism, but rather supplies it with ready-made food material, and may thus act as a stimulus to development. That the increased protein content of colostrum may so act is borne out by Birk's(211) observations. He showed that if an infant is fed upon human colostrum it shows a positive nitrogen balance even although it loses weight during the first few days after birth. If however the normal milk of a wet-nurse is supplied, and not colostrum, the nitrogen balance is negative, even although the infant is receiving the milk of its own species.

The Berlin data obtained for the purpose of the report already referred to showed that the weight-curves of infants fed upon the breast and upon boiled (just raised to 100° C.) milk, increased at approximately the same rate per cent., except during the early weeks of life. It was shown that the marked divergence of the curves at this period could almost certainly be attributed to the method of feeding.

The investigation of the biological properties of milk carried out in this report shows that the weight of evidence suggests the absence of any direct value in the biological substances *per se*, but it also most decidedly shows the paramount importance of providing breast milk for the young animal. It would seem impossible to emphasise this fact too strongly, and all those concerned in the health of infants should aim at obtaining satisfactory breast-feeding for all infants during, at any rate, the early weeks of life.

I am indebted to the Governing Body of the Lister Institute for permission to carry out the original work of the report in the Bio-Chemical Laboratory, and also to Dr. Harden and others for assistance in connection with the work, and take this opportunity of tendering them my thanks.

ADDENDUM.

An account of the work upon the "Biological Properties" of milk would hardly be complete at the present time without a brief reference to the work which has recently been done, and which is still in progress, upon the etiology of beri-beri. This disease, which appears to belong to the "Scurvy" group, is now known to be a disease of deficiency.

The work of Schaumann (Arch. f. Schiffs-und Troppen-Hygiene, May 1912) and of Funk (Journ. of Phys. 1912 and Journ. of State Medicine, June 1912) has shown that the disease can be cured even when the symptoms are well-marked and severe, by the administration of a substance "Vitamine" which has been isolated by Funk from rice-polishings and other materials. This substance is found in a large number of, if not in all, vegetable tissues, and appears to have slightly different properties in the different varieties. It is stated to be thermo-labile, but this property is believed by Funk to be different in the vitamines prepared from different tissues. At present the available data upon this point are somewhat indefinite.

It has been suggested that infantile scurvy may be caused by the deficiency of this substance in milk, it having presumably been destroyed by heat. Funk has prepared this substance from the variety of dried milk known as "Trumilk," so that there is no doubt that it is present in milk. Further, it is almost certain, from our knowledge of beri-beri, that this substance is essential for the well-being of the individual, so that it is presumably necessary for the infant. On this hypothesis the cure of infantile scurvy by the administration of vegetables would be accounted for. It is not unlikely that infantile scurvy may be produced by the deficiency of this substance in milk, either because the milk itself was deficient in the body, or because it had been destroyed by prolonged boiling or by a high temperature.

Before any statement can be made upon the subject a quantitative method for the estimation of the body must be discovered, the length of time of heating and the temperature required for its destruction must be known, and it would also be of value to know the effect upon the substance of keeping, in view of the fact that some of the cases of infantile scurvy appear to have been caused by feeding upon sterilised milk which had been kept.

Further the disease itself should be shown to be curable by the administration of the substance prepared from milk.

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THE MECHANISM OF PHAGOCYTOSIS FROM THE ADSORPTION POINT OF VIEW.

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With 22 charts.

INTRODUCTION.

DURING recent years many attempts have been made to show that the interactions of antigen and antibody may in large measure be explained on a basis of physical adsorption. The immunity processes which have been most studied from this point of view have been the toxin-antitoxin reaction, the adsorption of agglutinin by bacteria and the lysis of red blood corpuscles by specific sera and lytic substances of colloidal nature.

To discuss present opinion with regard to the nature of these reactions, would be out of place here, as in this communication I shall be concerned mainly with those processes which culminate in the phagocytosis of bacteria by the polymorphonuclear leucocytes.

There is a growing opinion, not yet perhaps resting on any extensive experimental evidence, that not only the sensitisation of an organism by a specific serum, but also, possibly, the mechanism by which the sensitised organism penetrates the limiting layer of the leucocyte wall, are essentially interactions between membranes of colloidal constitution and consequently amenable to the laws of colloidal chemistry.

Before an organism can reach the interior of the phagocyte, it must have passed through at least three preparatory phases :

(1) In the first place the organism must be efficiently sensitised by the opsonin in the serum (normal or specific). The removal of opsonin from a specific serum has not so far been studied by quantitative methods with a view to determining the numerical relations

subsisting between the opsonin adsorbed and the opsonin free after equilibrium has been attained. The removal of agglutinin by bacteria has been investigated from this standpoint by several observers (Eisenberg and Volk, Arrhenius, Bilz, Craw, Dreyer and Douglas, &c.), and although complete agreement has not yet been reached, it would appear at least that the balance of evidence supports the view that the reaction is more accurately explained on a basis of physical adsorption than on the more physico-chemical conception set forth by Arrhenius who applied to it the partition law in a modified form. In this communication some experiments will be detailed, the results of which go to show that the quantitative relations in the case of the removal of opsonin by bacteria are most accurately satisfied by equations of the adsorption type $\frac{y}{a} = kx^n$ (where y is the amount adsorbed, x the amount free in equilibrium and k, n and a are constants).

The question of so-called spontaneous phagocytosis without the intervention of a normal or specific serum, though of very great interest, is not considered here, but the fact that such spontaneous phagocytosis is influenced by the salt content of the menstruum suggests that in the process of opsonisation by serum, alterations in the electrical charge on the bacterial membrane may be an essential factor. We know from the work of Bechhold, Buxton and Teague and others that the so-called agglutinin-bacteria which have been exposed to an agglutinating serum and later freed therefrom by dialysis are more readily susceptible to precipitation by electrolytes than bacteria not so treated. In the case of opsonisation the "opsonin-bacteria" enter the second phase which doubtless corresponds to the second phase of agglutination, viz., the aggregation of the agglutinin-bacteria.

(2) In the second phase the sensitised organisms congregate at the periphery of the leucocyte. The writer (Ledingham 1906) in the course of experiments on the influence of temperature on phagocytosis, observed this attachment of sensitised bacteria to the leucocytes. At low temperatures where little or no phagocytosis occurred, this ring-distribution of the sensitised bacteria was most clearly seen. The suggestion was then made that once this phase of attachment had occurred, surface tension alterations between the bacterial- and leucocyte-membranes became the determining factors in phagocytosis. It has since been shown by Levaditi and Mutermilch (1910) that a similar aggregation of trypanosomes (Nagana) takes place in the neighbourhood of the leucocytes in the presence of a specific trypanolytic serum. When

phagocytic activity is paralysed by cold, this phase of aggregation or attachment nevertheless occurs. In the opinion of these authors, the attachment phase is to be regarded as a physico-chemical phenomenon analogous to agglutination. That a leucocyte is attracted by a sensitised trypanosome and goes out to look for it is in their opinion improbable. They regard it as far more likely that the actual attachment of the sensitised organism to the leucocyte results from a purely fortuitous *rencontre* of organism and phagocyte. With this opinion the writer agrees. The authors rightly point out however, that in the phenomenon of attachment there is a certain element of specificity involved. Thus (1) only sensibilised trypanosomes become attached, and (2) the attachment is only to cells of the leucocyte group and not to tissue cells such as hepatic or renal cells. The fact that only sensitised organisms become attached to leucocytes does not appear to the writer to require explanation on a specific basis. In the discussion of the first phase of sensitisation, I have pointed out that the opsonin-bacteria, like the agglutinin-bacteria, are probably in a more precipitable condition than non-sensitised bacteria. They are, in fact, as I have frequently observed, in a condition of extremely fine aggregation and if the sensitising fluid has marked agglutinating powers, the sensitised organisms are frankly clumped. That, under these conditions, therefore, sensitised organisms should be more readily cleared from the field by leucocytes than non-sensitised organisms is quite explicable on purely physical grounds. The leucocyte-membranes, with their well-known adhesive properties, clear the field probably much in the same way as coagulated egg white clarifies a turbid broth.

The fact that only cells of the leucocyte group play a part in the attachment phase, is difficult to account for on purely physical grounds. The explanation may however be found in the assumption that great variations occur in the constitution of the limiting membranes or adsorptive surfaces of cells. Under certain circumstances for example, it is known that fixed tissue cells such as hepatic cells or nerve cells may submit to penetration by foreign objects and on the other hand even among cells of the leucocytic group we know that the eosinophile cell does not, to any appreciable extent, act as a phagocyte of sensitised micro-organisms. Also among the frankly phagocytic cells such as the polymorphonuclear leucocytes, there is evidence that one cannot postulate a uniform physiological constitution of their adsorptive surfaces. We know, for example, that the immature polynuclear cells in Leukaemia have little phagocytic power probably in virtue of an incompletely developed adsorptive surface (Ledingham, Bushnell, &c.), and as we shall see later, differences in the constitution of the limiting layers of amoebae have been held to afford an explanation of the differences they exhibit in the mode of ingestion of foreign particles (Rhumbler). This phase of attachment has also been studied recently by Barikine (1911) working with leucocytes and sensibilised red cells. This observer who attacked the problem experimentally, made use of an interesting phenomenon (previously studied by Ponder in this country), viz., the adhesion of polymorphonuclear cells to glass plates. The strength of this adhesion, he attempted to measure by the amount of a jet of saline of fixed volume and height, necessary to effect their detachment from the plates. Only sensibilised red cells became in turn attached to these leucocytes adhering to the glass plates and very considerable jets of saline were required to wash off these red cells once they had secured an attachment to the leucocytes. When the leucocytes had been previously heated, they did not adhere to glass plates nor did sensibilised red cells attach themselves to such damaged leucocytes.

In the experiments of Levaditi and Mutermilch, heated leucocytes did become attached to sensibilised trypanosomes. I have myself observed in certain unpublished experiments with heated leucocytes and staphylococci, that the phase of attachment nevertheless took place. Barikine suggested that the heat employed had altered the consistence of the limiting membrane of the leucocyte.

(3) I come now to the third phase, viz., the mechanism of inclusion or incorporation within the body of the phagocyte.

The actual phagocytic act has long been regarded as essentially a vital phenomenon in which the amoeboid activity of the leucocyte plays an important part, but in recent years evidence has accumulated showing that as a factor in phagocytosis amoeboid activity has been greatly overrated. In the paper quoted above, the writer carried out certain experiments the results of which suggested that, provided the leucocyte was supplied with sensitised organisms, very little difference took place in the phagocytic intake whether the incubation was carried out at 18° C. or at 37° C. In some as yet unpublished experiments by Priestley in this Institute it has appeared that leucocytes supplied with sensitised organisms and kept at very low temperatures may, if given sufficient time, take up a progressively increasing number of organisms. The suggestion, therefore, is that at low temperatures the velocity of reaction (velocity of intake) may undoubtedly be slower than at 37° C. but the ultimate intake when sufficient time for equilibrium has been given, may approximate to that at 37° C. This accords with what we know of physical adsorption, *e.g.*, in the adsorption of dyes by filter paper.

Again it would seem that the phenomenon of amoeboid activity has received undue consideration from the manner in which it has been studied. In the course of a discussion of the *rôle* played by amoeboid activity in the life of the leucocyte, by Ullmann (1911), the fact is pointed out that the free-swimming polynuclear leucocyte is a perfectly round drop-like body and that it spreads itself out only in response to the stimulus caused by touching ground, *e.g.*, on the glass slide. Moreover, the protrusion of pseudopodia occurs in response only to abnormal stimuli and the wandering leucocyte may traverse large areas and thick layers of red blood corpuscles without sending out a single pseudopodium. That amoeboid activity as such is not essential to the phagocytic act is proved by the fact that in the performance of experiments in which mixtures of serum, leucocytes and micro-organisms are incubated together, very vigorous shaking can be employed without affecting the phagocytic activity of the leucocyte. In all experiments recorded in this communication the tubes containing the various mixtures were placed in a to-and-fro shaker working vigorously in an incubator kept at 37° C. Under these conditions it is difficult to suppose that independent leucocytic movements played any part at all in the process of phagocytosis.

The actual mechanism of entrance of red blood corpuscles into leucocytes has been studied microscopically by various observers and it would appear that most usually the impression obtained is that the leucocytic wall rises up leaving a meniscus in which the impinging red cell rests. Gradually the enveloping walls again coalesce and the red cell is imprisoned. A similar process of feeding takes place in the case of certain amoebae (Nahrungsaufnahme durch Umfliessung) (Rhumbler 1910). In amoebae also, food particles may be taken in, without any apparent movement on the part of the outer wall of the cell. The foreign body after contact with the cell wall appears to insinuate itself into the body of the phagocyte without any movements on the part of the latter (Nahrungsaufnahme durch "Import"). In these two types of inclusion Rhumbler considers that the determining factor is a diminution in the surface tension at the point of contact of the wall of the phagocyte with the foreign body. In other amoebae with denser and more skin-like ectoplasm, the more complicated processes of circumvallation and invagination were observed, but all these variations in the method of ingestion could readily be explained on mechanical principles.

Object of the present experiments.

The phagocytosis by leucocytes of minute objects such as bacilli cannot readily be followed microscopically but it seemed that some light might be thrown on the mechanism by investigating the quantitative relationships of the bacilli included in the leucocytes and the bacilli left free in the surrounding fluid. The present communication contains experimental data on this subject together with similar data regarding the first phase in phagocytosis, viz., the sensitisation of the organism.

For experiments of this nature, it is of great advantage to have a sensitising fluid which gives consistent results over a long series of experiments. Such a fluid was obtained by accident in the course of another research in which various guinea-pig end-pieces were being investigated. From an apparently normal guinea-pig an end-piece was prepared by the CO_2 method[1], which was found to give an exceedingly high phagocytic index with *B. typhosus*. The height of the phagocytic index with this fluid as compared with other end-pieces and normal complement was such as to render it extremely useful in experiments demanding long ranges of phagocytic indices. The properties of this end-piece, particularly its behaviour on dilution and on inactivation, will be first briefly recorded. Throughout the period of six months during which the experiments proceeded this end-piece was kept in the cold room at 1°–2° C. and samples were taken from it as required.

Remarks on the effect of dilution and of inactivation by heat.

It has to be remembered that the pure end-piece corresponds to a dilution of 1 in 10 of the original guinea-pig serum from which it was derived. No appreciable phagocytic index was obtained when the end-piece was diluted lower than 1 in 8 (*i.e.*, 1 in 80 of original serum), and in this respect the end-piece behaved like an ordinary normal serum, in which a measurable opsonising effect usually ceases in dilutions considerably under 1 in 100.

The fall in the phagocytic index following dilution of the end-piece was, as all the charts show, extremely regular, and, throughout the greater part of the dilution range, the points corresponding to the indices lie practically on straight lines the inclinations of which to the axis x

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[1] The exact details of the method of preparation of the end-piece from normal guinea-pig serum will be found in a paper by Ledingham and Dean, this *Journal*, Vol. XI. No. 2. 1912.

vary only very slightly. To this regular behaviour of the end-piece on titration is doubtless to be attributed much of the consistency obtained by its use in the experiments recorded in this communication. That its opsonising value ceased at such a low dilution as 1 in 8 was really no disadvantage. Ranges of sufficient length could readily be obtained by shorter spacing.

In its reaction to heat, the end-piece behaved quite differently from a normal serum. Heating for 30 minutes at 55° C. caused only a very

Influence of dilution on the sensitising fluid used in the experiments (end-piece).

Experiment I. (14. x. 11.)

Scheme: 3 vols. Serum + 2 vols. Leucocytes + 1 vol. Bacillary emulsion.
Strength of Bacillary emulsion 780,000 per cub. mm.
Incubation: 14 mins. at 37° C. in shaker.

Result: (see Chart I, *A*).

Dilution	Bacilli per leucocyte
1 in 1	20·01 = $\frac{1101}{55}$
1 in 2	6·62 = $\frac{709}{107}$
1 in 4	0·40 = $\frac{49}{121}$
1 in 8	0·02 = $\frac{2}{86}$
1 in 16	0·0 = $\frac{0}{74}$

Experiment II. (16. x. 11.)

Similar experiment to I. The end-piece was also heated (30 mins. at 55° C.) and then diluted like the unheated end-piece.

Incubation 12 mins.

Strength of emulsion 1,400,000 per cub. mm.

Result: (see Chart I, *B* and *C*).

Dilution	Unheated End-piece Phagocytic Index	Heated End-piece Phagocytic Index
1 in 1	15·4 = $\frac{1203}{78}$	13·7 = $\frac{1440}{105}$
1 in 2	2·25 = $\frac{459}{204}$	0·65 = $\frac{161}{248}$
1 in 4	0·097 = $\frac{27}{277}$	0·043 = $\frac{16}{368}$
1 in 8	0·01 = $\frac{4}{234}$	0·01 = $\frac{2}{179}$

slight fall in the opsonising value (Exp. II) and even 30 minutes heating at 60° C. had the effect only of bringing the phagocytic index down to a little less than half that of the unheated end-piece. Heating at 65° C. for 30 minutes still left a very small residuum but after heating at 70° C.

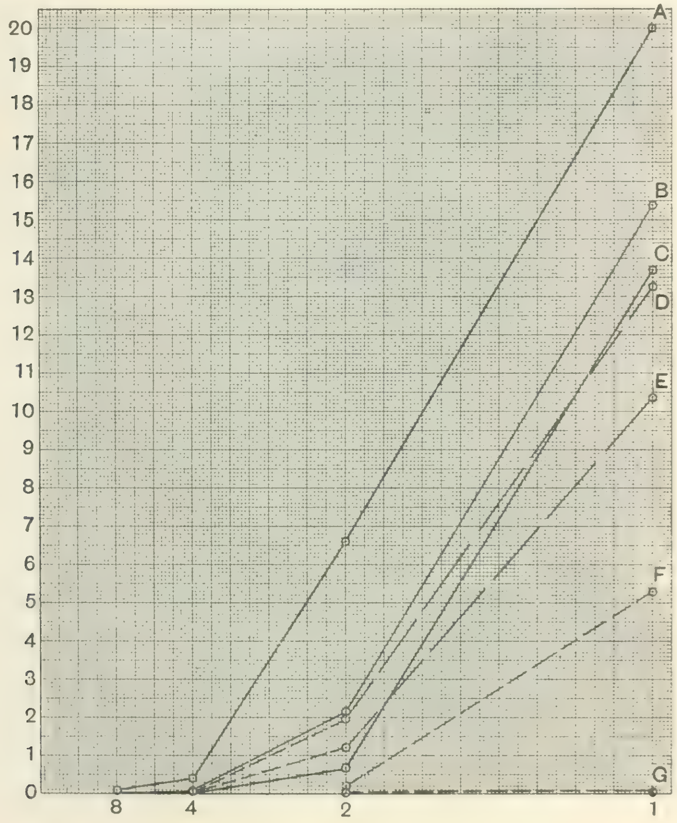

Chart I.

Experiment III. (18. x. 11.)

The end-piece was on this occasion heated at various temperatures for 30 mins. Dilutions were then made.

Result: (see Chart I, *D*, *E*, *F*, *G*).

Dilution	Unheated E. Phag. Index	E. 55° C. Phag. Index	E. 60° C. Phag. Index	E. 65° C. Phag. Index	E. 70° C. Phag. Index
1 in 1	13·27	10·38	5·32	0·054	0·018
1 in 2	1·98	1·21	0·4	0·021	0·00
1 in 4	0·19	0·09	0·025	0·01	0·00

practically no phagocytic values could be obtained (Exp. III). This particular end-piece, therefore, in so far as its opsonin-content was concerned, was remarkably thermostable and might well have been derived from an ordinary immune serum.

On the specificity of the end-piece employed in the experiments.

An attempt was made to determine whether the end-piece behaved, in an adsorption process, like an immune typhoid serum.

The following mixtures were prepared :

3 vols E. + 1 vol. Saline + 1 vol. of thin emulsion of *B. typhosus*,
„ „ „ „ „ *Staph. aureus.*

These were digested at room temperature for ten minutes and then centrifugalised completely. The supernatants were tested in the ordinary way, against both *Staph. aureus* and *B. typhosus.*

3 vols. E. + 2 vols. Saline constituted the control unadsorbed fluid.

Result :

	S. aureus Phag. Index	*B. typhosus* Phag. Index
Control unadsorbed	0·03	7·5
Supernatant after adsorption with *B. typhosus*	0·00	0·05
Supernatant after adsorption with *Staph. aureus*	0·00	6·1

The experiment shows very clearly that adsorption with *B. typhosus* removes the typhoid antigen almost entirely, whereas adsorption with *Staph. aureus* has practically no influence on the specific typhoid-opsonins.

As I have already indicated, this end-piece came from a presumably normal guinea-pig in the course of a research in which numerous end-pieces from normal guinea-pigs were examined (Ledingham and Dean

Experiment IV. (25. xi. 11.)

Scheme: 2 vols. E. + 1 vol. Leucocytes + 1 vol. Bacillary emulsion.
Shorter spacing employed in preparing the dilutions of the end-piece.
Incubation 14 mins.

Result :

Dilution	Phagocytic Index
10 in 12	15·14
9 in 12	12·91
8 in 12	10·77
7 in 12	8·78
6 in 12	6·05
5 in 12	5·65

1912). Investigation of its properties might lead one to suppose that the animal from which it was derived had been in some way specifically immunised and that it had gained entrance to the normal stock by

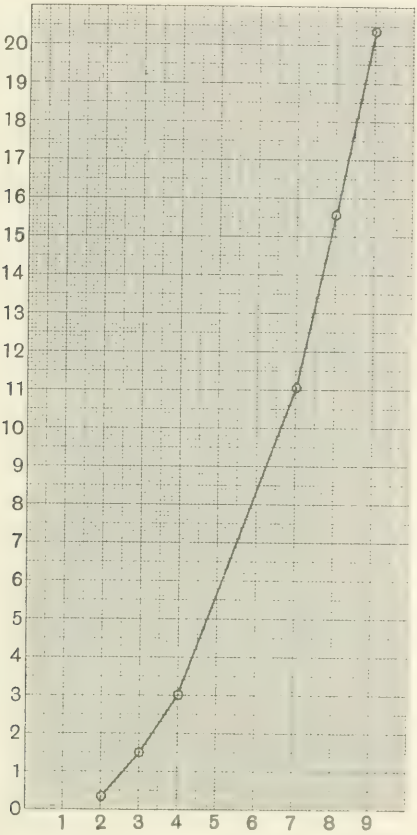

Chart II. *x*. Concentration of end-piece. *y*. Phagocytic index.

Experiment V. (18. xii. 11.)

Similar experiment to IV. Dilutions carried a little further.

Result: (see Chart II).

Dilution	Phagocytic Index
9 in 12	20·35
8 in 12	15·55
7 in 12	11·06
4 in 12	3·02
3 in 12	1·51
2 in 12	0·35

mistake. This is unlikely, but if so, the only immunisation it could have received, was a sub-lethal dose of typhoid bacilli not less than six months previously. The matter must remain at present unsettled.

I now pass to the main purpose of the experiments, viz., the determination of the numerical relationships subsisting between the adsorbed (*i.e.*, the phagocytosed) and the free (*i.e.*, not phagocytosed) bacilli in opsonic experiments. The subject was treated in three stages as follows:

Stage I. Experiments designed to show the relation between the adsorbed and the free bacilli, where the two processes of sensitisation and phagocytosis were allowed to go on simultaneously.

Stage II. Experiments on the nature of opsonic sensitisation from the adsorption point of view.

Stage III. Experiments on the nature of the union between the leucocyte and the sensitised bacilli suspended in salt solution.

STAGE I. (Exps. A, B and C.)

Technique of experiments.

A moderately thick emulsion of *B. typhosus* grown on agar for about 24 hours at 25° C. was taken and successive dilutions prepared therefrom. The number of bacilli in a convenient dilution was estimated directly with a Thoma-Zeiss apparatus. The number of leucocytes per cubic millimetre in the human blood-leucocyte emulsion was estimated by the same method. With these data, it was easy to calculate the initial ratios of bacilli to leucocytes in each cubic mm. of the incubated fluids. This ratio (denoted B/L in all experiments quoted) expresses simply the number of bacilli available for each leucocyte at the commencement of incubation.

The incubated fluid generally consisted of 1 vol. End-piece, 1 vol. Bacillary emulsion and 1 vol. Leucocyte emulsion (human). These were drawn up into a large-bored capillary pipette with rubber teat from which they were immediately ejected into a small test-tube and there thoroughly mixed by aid of the pipette. The tube was then placed in the to-and-fro shaker working in the incubator at 37° C.

At the end of the period of incubation the test-tubes were withdrawn from the shaker in the order in which they went in. By means of a capillary pipette a small sample from each test-tube was immediately placed on a slide and a film prepared. This was fixed by methyl alcohol and stained by Giemsa's solution.

Experiment A. (9. XI. 11.)

Strength of bacillary emulsion = 11,212,800 per cub. mm. (calculated from a convenient dilution).

Strength of leucocyte emulsion = 6,180 polymorphonuclear leucocytes per cub. mm.

Result:

B/L	P. I. ("Amount Adsorbed")	Percentage "Adsorbed"	Percentage "Free"
1814·36	?	?	?
907·18	$1{\cdot}96 = \frac{401}{204}$	0·21	99·79
453·59	$2{\cdot}18 = \frac{445}{204}$	0·48	99·52
226·79	$3{\cdot}28 = \frac{670}{204}$	1·44	98·56
113·39	$9{\cdot}25 = \frac{1887}{204}$	8·15	91·85
56·69	$13{\cdot}59 = \frac{1849}{136}$	23·97	76·03
28·34	$15{\cdot}89 = \frac{2162}{136}$	56·06	43·94
14·17	$8{\cdot}84 = \frac{1199}{136}$	62·38	37·62
7·08	$6{\cdot}53 = \frac{1111}{170}$	92·23	7·77

Experiment B. (11. I. 12.)

Strength of bacillary emulsion = 11,944,000 per cub. mm.
,, ,, leucocyte ,, = 8,000 ,, ,,
Incubation time 11 mins.

Result:

B/L	P. I. ("Amount adsorbed")	Percentage "Adsorbed"	Percentage "Free"
1493·0	$0{\cdot}11 = \frac{36}{317}$	0·007	99·92
746·5	$0{\cdot}087 = \frac{24}{275}$	0·011	99·99
373·25	$0{\cdot}33 = \frac{66}{199}$	0·088	99·91
186·62	$1{\cdot}79 = \frac{426}{238}$	0·95	99·05
93·31	$5{\cdot}89 = \frac{1403}{238}$	6·31	93·69
46·65	$9{\cdot}69 = \frac{1318}{136}$	20·77	79·23
23·32	$7{\cdot}02 = \frac{955}{136}$	30·09	69·91
11·66	$5{\cdot}30 = \frac{721}{136}$	45·45	54·55
5·83	$2{\cdot}95 = \frac{689}{233}$	50·60	49·40

Experiment C. (5. II. 12.)

Strength of bacillary emulsion = 28,672,000 per cub. mm.
,, ,, leucocyte ,, = 7,000 ,, ,,
Incubation time 13 mins.

Result:

B/L	P. I. ("Amount Adsorbed")	Percentage "Adsorbed"	Percentage "Free"
4096	? < 0·01	?	? 99·999
2048	? < 0·06	?	99·999
1024	0·099 = $\frac{18}{181}$	0·0096	99·990
512	0·306 = $\frac{90}{297}$	0·059	99·941
256	2·10 = $\frac{371}{176}$	0·82	99·18
128	8·33 = $\frac{1326}{159}$	6·51	93·49
102·4	9·56 = $\frac{1301}{156}$	9·33	90·67
85·3	10·04 = $\frac{1707}{170}$	11·77	88·23
64	12·02 = $\frac{2045}{170}$	18·78	81·22
51·2	12·27 = $\frac{1669}{136}$	23·96	76·04
32	13·55 = $\frac{1844}{136}$	42·34	57·66
16	10·27 = $\frac{1397}{136}$	64·18	35·82
8	5·14 = $\frac{874}{170}$	64·25	35·75
4	3·52 = $\frac{600}{170}$	88·0	12·0

As a rule the number of leucocytes counted in each case was well over one hundred and frequently counts of 200 or 300 were made. The actual number of leucocytes counted in each experiment will be quoted. The phagocytic index so obtained gave the average number of bacilli taken up by each leucocyte (denoted in the experiment by P. I., phagocytic index, or "Amount adsorbed").

By subtraction of P. I. from the initial ratio B/L, the number of bacilli left free outside the leucocyte was calculated ("Amount free").

Analysis of experiments A, B and C.

In each of these three experiments, the numerical results of which are quoted, there are three constants, viz., (1) the amount of sensitising fluid, (2) the number of leucocytes (*i.e.*, the "Adsorbent"), and (3) the period of incubation, and one variable, viz., the concentration of bacilli.

As the concentration of bacilli increases and consequently also the ratio B/L, the phagocytic index (or the amount "adsorbed" per leucocyte) increases up to a maximum. With further increase of the ratio B/L, the phagocytic index progressively falls. The course of events is, in fact, exactly analogous to what takes place when increasing quantities of antigen are added to a constant amount of a precipitating antiserum. It is possible, therefore, to choose two entirely different concentrations of bacilli (antigen) which with a constant quantity of antiserum will give similar phagocytic indices.

The fall in the index which occurs after the maximum is reached is obviously due to excess of antigen, the consequence being that the number of bacilli completely sensitised by the available antiserum continually diminishes and approaches the zero line in asymptotic fashion.

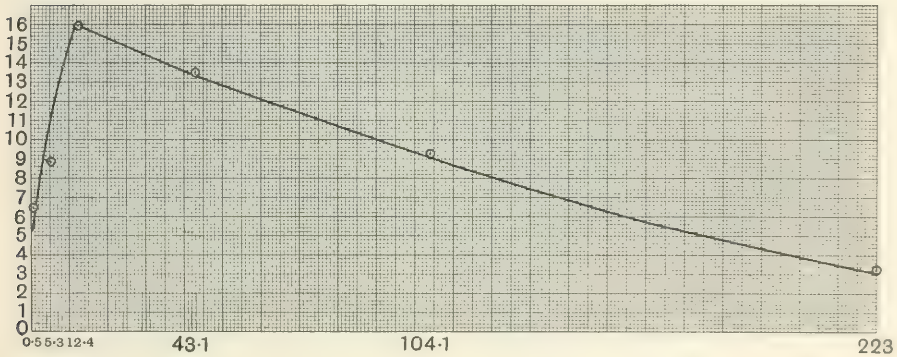

Chart III. x. Bacilli "free." y. Bacilli "bound."

In Charts III, IV, and V are shown portions of the curves obtained by plotting the "Total amounts adsorbed" (= P. Indices) against the "Total amounts free" (= B/L – P. I.) in the experiments A, B, C. The curves are similar in type, each consisting of two portions—one slightly concave and the other convex to the axis of x.

Although, however, it is impossible to find a relationship between the "Amount adsorbed" and the "Amount free" which will hold good throughout the whole extent of these discontinuous curves, a very definite relation is found to subsist between the initial concentration of bacilli and the "Percentage amount adsorbed" (or the "Percentage amount free").

As the initial concentration of bacilli increases, the "Percentage amount free" progressively increases and approaches the value 100

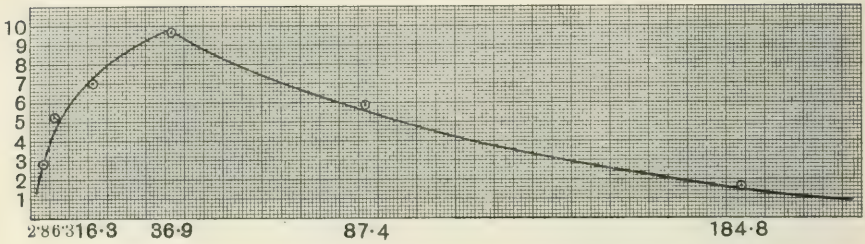

Chart IV. *x*. Bacilli "free." *y*. Bacilli "bound."

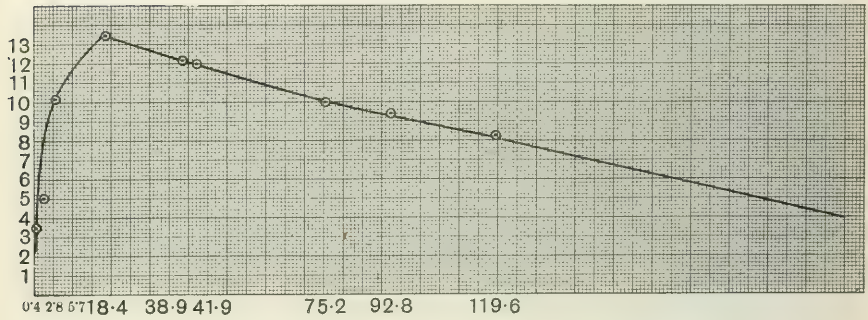

Chart V. *x*. Bacilli "free." *y*. Bacilli "bound."

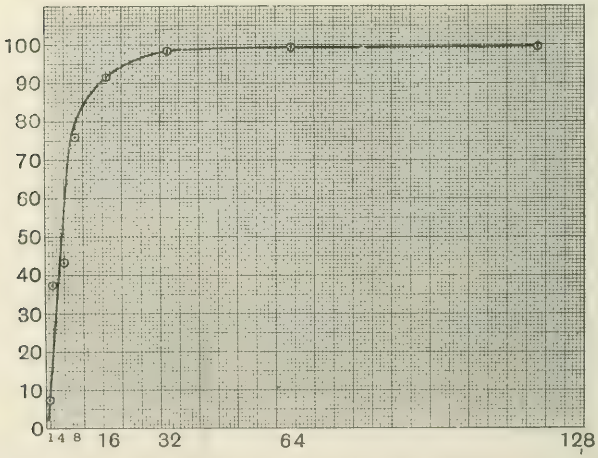

Chart VI. *x*. Initial concentration of bacilli. *y*. Percentage of bacilli "free."

asymptotically. Or, as the initial concentration of bacilli diminishes, the "Percentage amount adsorbed" progressively increases until a point is reached at which (as in Exp. A) over 90 % of the available bacilli are taken up. In Charts VI, VII, and VIII, the initial concentrations of bacilli (from Exps. A, B, and C) are plotted against the "Percentage free" and it will be seen that in all three cases, the points so obtained lie on a smooth curve to which the line $y = 100$ is an asymptote.

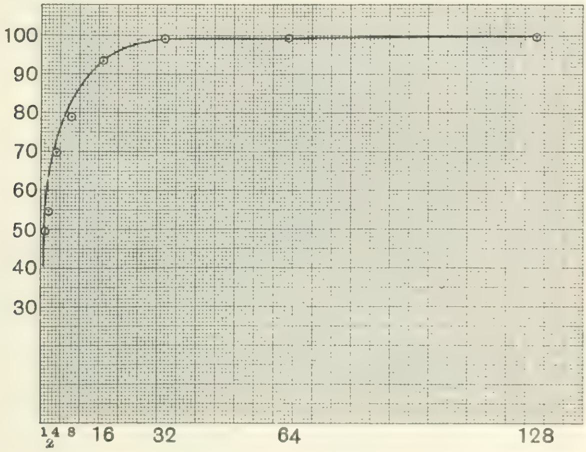

Chart VII. x. Initial concentration of bacilli. y. Percentage of bacilli "free."

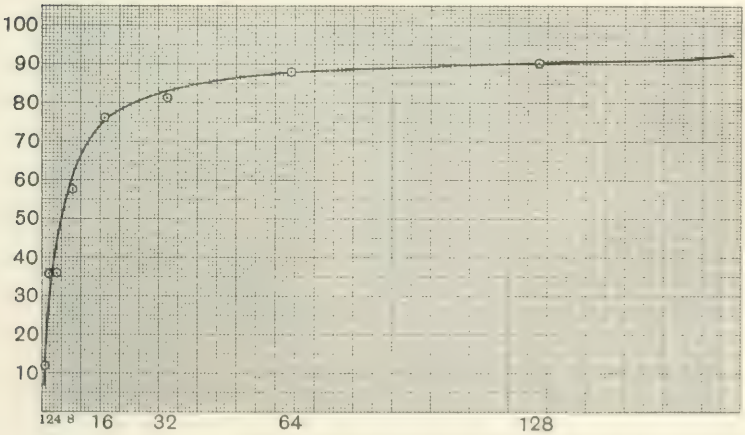

Chart VIII. x. Initial concentration of bacilli. y. Percentage of bacilli "free."

The curves are hyperbolic in type but I have been unable to find a satisfactory mathematical expression for them. The processes involved in this type of experiment are, however, extremely complicated as the two phases of sensitisation and phagocytosis (both probably of an adsorption character) proceed simultaneously and the time is constant. The limitation of the period of incubation to 10–12 minutes is necessary in order to avoid intracellular lytic changes which would interfere with accurate enumeration.

In the following two stages the phenomena of sensitisation (opsonisation) and of *phagocytosis of sensitised organisms* are treated separately.

STAGE II.

Opsonisation from the adsorption point of view.

The experiments bearing on this question took the following general type. A series of dilutions of the sensitising fluid (end-piece) in saline were prepared. Each of these dilutions was digested for a certain time at a certain temperature, with a constant number of bacilli. At the end of the period of digestion, the tubes were completely centrifugalised and the supernatant fluids collected. The relative amounts of opsonin left in each of these supernatants were then estimated by means of the phagocytic index, a fresh typhoid emulsion being employed for this purpose. These phagocytic indices represented the relative amounts of opsonin "free." The relative amounts of opsonin "bound" were ascertained by subtracting the phagocytic indices of the various supernatants from the indices of the corresponding undigested dilutions, all being tested at the same time.

In adopting this value for the bound opsonin it is fully realised that the fall in the phagocytic index which occurs on simple dilution of the sensitising fluid is not a linear function of the concentration. In the three experiments now to be quoted, the curve of the indices when plotted against the serum-concentrations as abscissae, is in the greater part of its extent a straight line which however bends round towards the origin as the axis of x is approached. I have calculated in two of these cases (Exps. E and F) that the closest approximation to these dilution curves (see the upper curves in Charts XI and XIII) is a parabolic curve of the form $x^2 = ky$, where x represents the concentration of the serum, y the phagocytic index and k is a constant. The actual value of the exponent of x was calculated in these two cases to be 2·1 and 2·3 which figures gave excellent values for the corresponding constants.

The lower curves in these charts, representing the indices of the supernatants, are also of logarithmic type but the exponents of x are as might be expected from their appearance much higher. In the three cases quoted the values of n were 6·1, 10·0 and 3·3 respectively.

Experiment D. (25. XI. 11.)

The following digestions were carried out for 55 mins. at 37° C. and 85 mins. at room temperature. (Total volume in each 0·6 c.c.)

1. 0·5 c.c. E. + 0 c.c. saline + 0·1 c.c. bacillary emulsion.
2. 0·45 c.c. E. + 0·05 c.c. saline + do. do.
3. 0·4 c.c. E. + 0·1 c.c. saline + do. do.
4. 0·35 c.c. E. + 0·15 c.c. saline + do. do.
5. 0·30 c.c. E. + 0·2 c.c. saline + do. do.
6. 0·25 c.c. E. + 0·25 c.c. saline + do. do.

After centrifugalisation, the supernatants were collected and their phagocytic indices estimated. At the same time the indices of the following unadsorbed dilutions were estimated.

1. 0·5 c.c. E. + 0·1 c.c. saline.
2. 0·45 c.c. E. + 0·15 c.c. saline.
3. 0·4 c.c. E. + 0·2 c.c. saline.
4. 0·35 c.c. E. + 0·25 c.c. saline.
5. 0·3 c.c. E. + 0·3 c.c. saline.
6. 0·25 c.c. E. + 0·35 c.c. saline.

(Total volume in each 0·6 c.c.)

Result :

Concentration of sensitising fluid. E.	P. I. before adsorption	P. I. after adsorption or "Amount Free"	Amount "Bound"	Percentage "Bound"
10 in 12	$15{\cdot}141=\frac{2574}{170}$	$5{\cdot}129=\frac{1108}{216}$	10·012	66·12
9 in 12	$12{\cdot}911=\frac{1317}{102}$	$3{\cdot}447=\frac{586}{170}$	9·464	73·3
8 in 12	$10{\cdot}779=\frac{1466}{136}$	$1{\cdot}352=\frac{230}{170}$	9·427	87·4
7 in 12	$8{\cdot}784=\frac{896}{102}$	$0{\cdot}7563=\frac{180}{238}$	8·027	91·3
6 in 12	$6{\cdot}054=\frac{1120}{185}$	?0·2	?5·854	?96·6
5 in 12	$5{\cdot}654=\frac{803}{142}$	$0{\cdot}0867=\frac{19}{219}$	5·567	98·4

It will be seen that, as the concentration of the sensitising fluid diminishes, the percentage amount of opsonin bound progressively increases.

In Chart IX the curves of the phagocytic indices of the supernatants and of the unadsorbed serum-dilutions, respectively, have been plotted, and in Chart X are shown the points obtained by plotting the "bound" against the "free" opsonin. On the same chart the logarithms of these latter values are plotted and it will be seen that a straight line can be made to pass very closely through them.

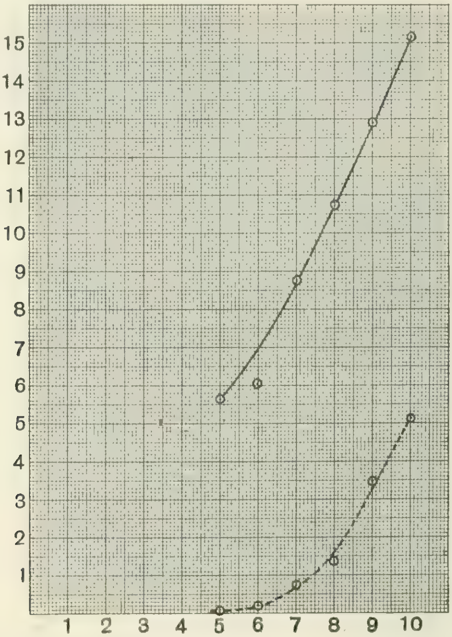

Chart IX. x. Initial concentration of end-piece. y. Phagocytic Indices. Upper curve unadsorbed. Lower curve supernatant.

Analysis. Assuming that the union of opsonin and bacilli is of the nature of an adsorption process, expressible by the equation $Y = Kx^n$, where Y represents the "amount bound," x the "amount free" (in equilibrium) and K and n are constants, the best value of "n" in the equation $\log Y = \log K + n \log x$ is deduced in the following way:

Bound $(Y)\times100$	Free $(X)\times100$	Log. Bound (y)	Log. Free (x)
1001·2	512·9	3·005181	2·700033
946·4	344·7	2·976075	2·537441
942·7	135·2	2·974374	2·130977
802·77	75·63	2·904553	1·878694
?585·4	?20·00	2·767453	1·30103
556·73	8·67	2·745621	0·938019

Mean of x = 1·914365667. Mean of y = 2·895542833.

Deviations from means:

x^1	y^1	x^1y^1	$(x^1)^2$
+ ·7856673	+ ·10963817	+ ·086139	·6172731
+ ·6230753	+ ·0805322	+ ·0501776	·3882228
+ ·21661133	+ ·078831167	+ ·0170757	·0469205
− ·0356717	+ ·0090102	− ·0003214	·0012725
− ·61333567	− ·1280898	+ ·078562	·3761806
− ·97634667	− ·149921833	+ ·1463757	·9532528

$$\Sigma(x^1y^1) = 0{\cdot}3780086, \qquad (x^1)^2 = 2{\cdot}3831223, \qquad n = \frac{\Sigma(x^1y^1)}{(x^1)^2} = 0{\cdot}1586.$$

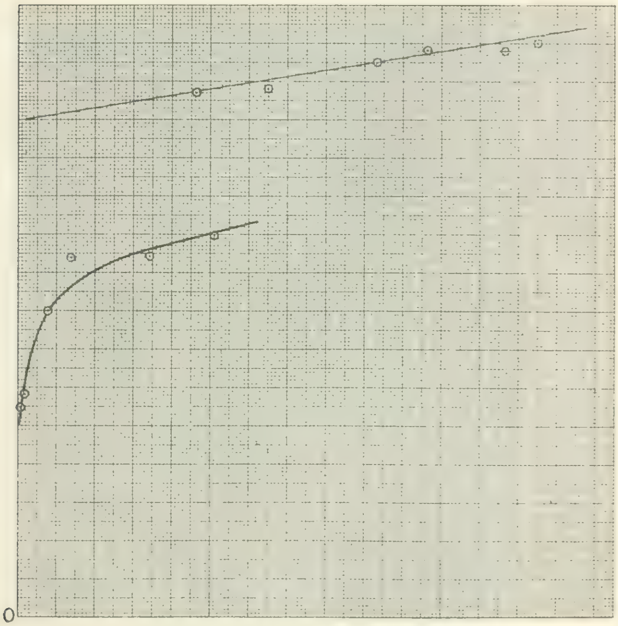

Chart X. *x.* Log. Opsonin "free." *y.* Log. Opsonin "bound." (Upper curve.)
x. Opsonin "free." *y.* Opsonin "bound." (Lower curve.)

The value 0·158 for the exponent "n" gives the following values for log K^1:

2·578	and for K^1	378
2·575		375
2·637		433
2·607		404
2·561		363
2·597		395

As the figures for the "bound" and "free" have been multiplied by 100, the values of log K in the equation $y = kx^n$ are calculated from the following equation:

$$\log K = \log K^1 + 2\,({\cdot}158) - 2.$$

These values of log K are therefore (with $n = 0{\cdot}158$)

0·894	and for K 7·83
0·891	7·78
0·953	8·97
0·923	8·37
0·877	7·53
0·913	8·18

An equation therefore of the adsorption type $Y = Kx^{0\cdot158}$ will express very satisfactorily the relation in equilibrium of the bound to the free opsonin.

Experiment E. (18. XII. 11.)

Successive dilutions of the sensitising fluid were digested as in the last experiment, with a fixed volume of bacillary emulsion.

Period of digestion 45 minutes at 37° C.

After digestion the tubes were immediately centrifugalised. The phagocytic indices of the supernatants were then estimated and compared with those of the corresponding unadsorbed dilutions.

Result:

Concentration of sensitising fluid. E.	P. I. before adsorption	P. I. after adsorption or "Amount Free"	Amount "Bound"	Percentage "Bound"
9 in 12	$20{\cdot}35 = \frac{1791}{88}$	$1{\cdot}69 = \frac{461}{272}$	18·66	91·2
8 in 12	$15{\cdot}55 = \frac{2115}{136}$	$0{\cdot}63 = \frac{193}{306}$	14·92	95·9
7 in 12	$11{\cdot}06 = \frac{1129}{102}$	$0{\cdot}128 = \frac{30}{233}$	10·93	98·8
4 in 12	$3{\cdot}02 = \frac{417}{138}$	$0{\cdot}0266 = \frac{4}{150}$	2·993	99·1
3 in 12	$1{\cdot}51 = \frac{290}{192}$	?		
2 in 12	$0{\cdot}356 = \frac{99}{278}$	?		

Obviously, in the above experiment, the amount of bacilli used for digestion with the serum dilutions, was too large to permit a suitable range of "free-opsonin" values capable of accurate estimation. Even in the case of the highest concentration of serum, the digestion effected a removal of over 90 $^0/_0$ of the available opsonin. In spite of the short range, however, it is quite apparent that with falling concentration of serum the relative amount of opsonin removed, increases progressively.

In Chart XI are plotted the curves formed by the indices of the supernatants and the unadsorbed fluids respectively, and in Chart XII the "opsonin bound" is plotted against the "opsonin free." The points obtained by plotting the logs. of these quantities are shown on the same chart (Chart XII).

Analysis of Experiment E.

Bound × 100	Log. Bound	Free × 100	Log. Free
1866	3·270912	169	2·22788
1492	3·173769	63	1·79934
1093·2	3·03862	12·8	1·10721
299·34	2·476107	2·66	0·42488

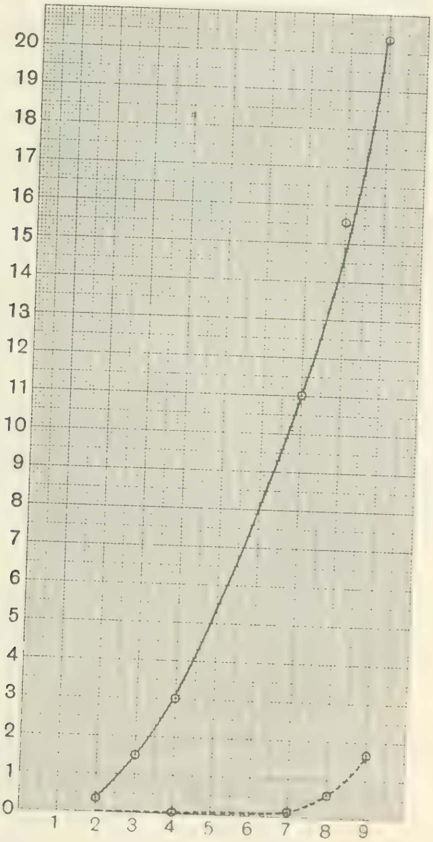

Chart XI. x. Initial concentration of end-piece. y. Phagocytic indices. Upper curve unadsorbed. Lower curve supernatant.

The value 0·217 for the exponent "n" gives the following values for log K:

1·221	and for K 16·64
1·217	16·48
1·222	16·68
0·917	8·26

That the fourth point is so far out, is in all probability explained by inaccuracy in estimating the corresponding low phagocytic index.

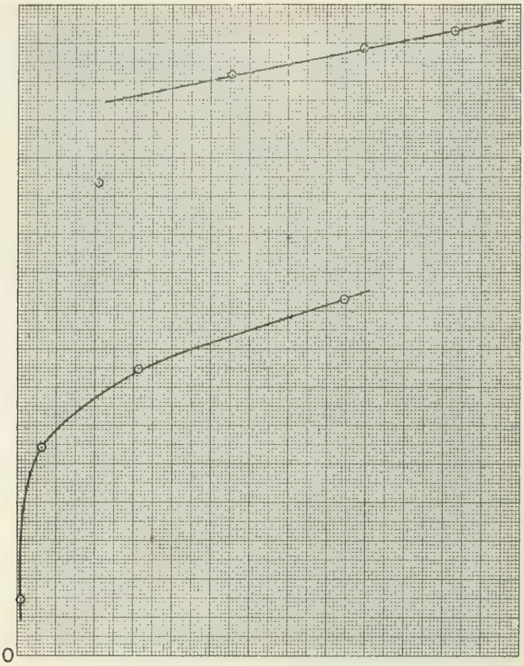

Chart XII. *x*. Log. Opsonin "free." *y*. Log. Opsonin "bound." (Upper curve.) *x*. Opsonin "free." *y*. Opsonin "bound." (Lower curve.)

Experiment F. (2. I. 12.)

On this occasion a very thin emulsion of bacilli was employed for digestion purposes. Technique in other respects similar.

Result:

Concentration of sensitising fluid. E.	P. I. before adsorption	P. I. after adsorption or "Amount Free"	Amount "Bound"	Percentage "Bound"
9 in 12	17·22	6·30	10·92	63
8 in 12	14·4	4·71	9·69	67
7 in 12	11·5	3·4	8·1	70
6 in 12	8·67	2·1	6·57	75
5 in 12	6·05	1·1	4·95	81
4 in 12	3·3	0·403	2·89	87
3 in 12	1·088	0·08	1·008	92
2 in 12	0·76	0·038	0·722	95

In Chart XIII are plotted the curves formed by the indices of the supernatants and the corresponding unadsorbed fluids and in Chart XIV the curve connecting the "opsonin bound" with the "opsonin free" in equilibrium has been plotted. On the same chart are also shown the points obtained by plotting the logarithms of these quantities.

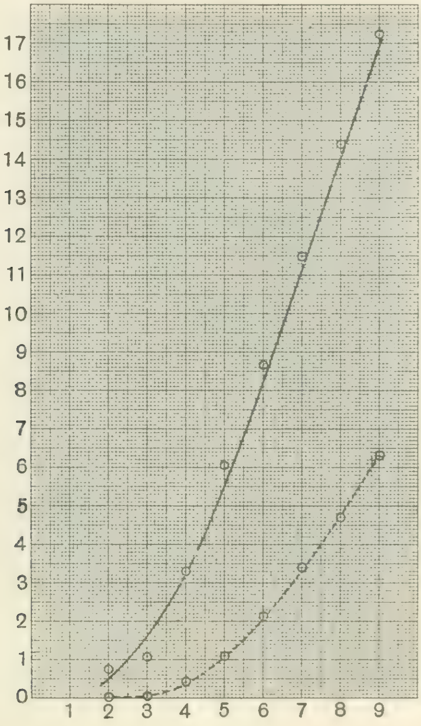

Chart XIII. *x*. Initial concentration of end-piece. *y*. Phagocytic indices. Upper curve unadsorbed. Lower curve supernatant.

Analysis of Experiment F.

Bound × 100	Log. Bound	Free × 100	Log. Free
1092	3·0382	630	2·7993
969	2·9863	471	2·6730
810	2·9084	340	2·5314
657	2·8175	210	2·3222
495	2·6946	110	2·0413
289·7	2·4608	40·3	1·6053
100·8	2·003	8·0	0·903
6·2	0·792	3·8	0·5797

From Chart XIV it will be seen that the first six points fall almost exactly on a straight line, the 7th and 8th points (corresponding to the very low indices) being slightly out.

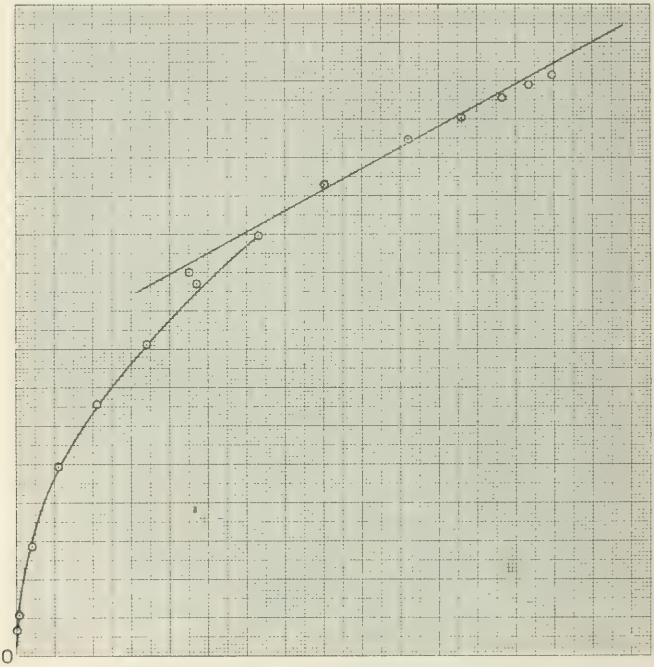

Chart XIV. *x*. Log. Opsonin "free." *y*. Log. Opsonin "bound." (Upper curve.)
x. Opsonin "free." *y*. Opsonin "bound." (Lower curve.)

The value 0·48 for the exponent "*n*" gives the following values for log K^1:

1·694	and for K^1 14·77
1·703	14·80
1·693	14·76
1·702	14·79
1·714	14·83
1·691	14·76
1·570	14·35
1·580	14·39

The agreement between these values of K^1 (with exception of the latter two, corresponding to the low indices) is exceedingly satisfactory.

Further experiments on adsorption of opsonin by bacilli.

Experiment G.

In this experiment a constant quantity of serum (end-piece) was digested with varying quantities of bacilli for a certain fixed time. The mixtures were then centrifugalised and the phagocytic indices of the supernatants tested in the ordinary way.

The original bacillary emulsion was found to contain 11,639,000 bacilli per cub. mm. From this emulsion various dilutions were prepared.

The following mixtures were digested for 1½ hours at room temperature and 45 minutes at 37° C.:

1.	0·4 c.c. end-piece	+	0·1 c.c. of original emulsion.	
2.	do.	+	do.	(diluted 1 in 2).
3.	do.	+	do.	(,, 1 in 4).
4.	do.	+	do.	(,, 1 in 8).
5.	do.	+	do.	(,, 1 in 16).
6.	do.	+	do.	(,, 1 in 32).
7.	do.	+	do.	(,, 1 in 64).
8.	do.	+	do.	(,, 1 in 128).
Control.	do.	+	0·1 c.c. Saline (unadsorbed).	

After digestion the tubes were centrifugalised and the supernatants collected. The phagocytic indices of these supernatants were then tested and compared with that of the unadsorbed end-piece.

Result:

			Phagocytic Index	Percentage "Free"
Unadsorbed serum ...	...	...	$5{\cdot}9 = \frac{806}{136}$	100·0
After adsorption with bacillary emulsion		(1 in 128)	$2{\cdot}8 = \frac{581}{204}$	47·4
do.	do.	(1 in 64)	$1{\cdot}5 = \frac{259}{170}$	25·4
do.	do.	(1 in 32)	$0{\cdot}81 = \frac{138}{170}$	13·7
do.	do.	(1 in 16)	$0{\cdot}5 = \frac{85}{170}$	8·4
do.	do.	(1 in 8)	$0{\cdot}72 = \frac{104}{144}$	12·2
do.	do.	(1 in 4)	$0{\cdot}63 = \frac{93}{147}$	10·6
do.	do.	(1 in 2)	$0{\cdot}32 = \frac{67}{204}$	5·4
do.	do.	(1 in 1)	$0{\cdot}43 = \frac{89}{204}$	7·2

In the annexed Chart XV the percentages of free opsonin have been plotted against the bacillary concentrations employed for adsorption. The curve so formed descends at first very rapidly towards the axis of *x*. It then proceeds very slowly and with some irregularity (points 5, 6 and 7) but a complete depletion of the opsonin is not attained at least within the limits of the present experiment.

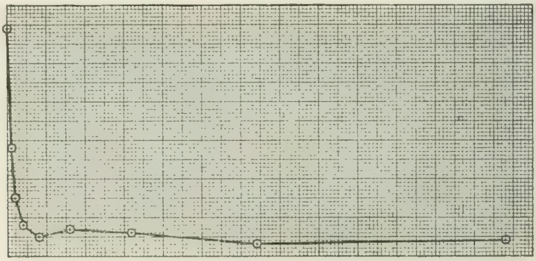

Chart XV. *x*. Concentration of bacilli used for adsorption.
y. Phagocytic indices of supernatants.

Experiment H.

On the rate of adsorption of opsonin by typhoid bacilli at 37° C. *and at* 12° C.

Adsorption at 37° C. The great rapidity with which opsonin is adsorbed by a bacillary emulsion, makes accurate technique difficult. In this experiment the following mixture was prepared, the various constituents having, before mixing, been raised to the temperature of the incubator (47° C.):

0·4 c.c. end-piece + 0·2 c.c. saline + 0·1 bacillary emulsion.

After admixture the tube was placed in the shaker working in the incubator at 37° C. Small samples were removed from the tube at various periods after mixing and immediately centrifugalised. No attempt was made, however, to maintain a temperature of 37° C. during the centrifugalisation process. After ten minutes in the centrifuge the clear supernatant fluids were pipetted off. The phagocytic indices of these were estimated and compared with that of the unadsorbed serum.

Apparently, therefore, no less than 80 °/₀ of the opsonin was removed practically during the short period required for thorough admixture of the bacilli with the serum. In ten minutes after admixture, a little over 90 °/₀ was removed but no further depletion took place although samples were tested up to 2½ hours after admixture. Equilibrium between the bound and free opsonin was reached in 10 minutes (approximately).

Result:

(Period in centrifuge not counted)	Phagocytic Index	Percentage Adsorbed
Before adsorption	11·26 = $\frac{1149}{102}$	-
1½ mins. after mixing	2·30 = $\frac{470}{204}$	79·5
5½ ,, ,,	1·53 = $\frac{314}{204}$	86·4
9½ ,, ,,	0·98 = $\frac{201}{204}$	91·2
18¼ ,, ,,	0·86 = $\frac{130}{151}$	92·3
31¾ ,, ,,	1·37 = $\frac{234}{170}$	87·8
49½ ,, ,,	1·28 = $\frac{175}{136}$	88·6
57¼ ,, ,,	1·27 = $\frac{174}{136}$	88·7
144 ,, ,,	1·01 = $\frac{207}{204}$	91·0
159½ ,, ,,	0·93 = $\frac{165}{176}$	91·7

Adsorption at 12° C. Technique similar to that employed in previous experiment. (See Chart XVI.)

Result:

	Phagocytic Index	Percentage removed
Unadsorbed serum	6·28	—
½ min. (*i.e.* immediately after mixing)	2·3	63·3
2 mins. after mixing	1·0	80·8
4½ ,, ,, ...	0·57	90·9
7½ ,, ,, ...	0·36	94·2
14¼ ,, ,, ...	0·31	95·0
22 ,, ,, ...	0·38	93·9
30 ,, ,, ...	0·17	97·2
57¾ ,, ,, ...	0·19	96·9
74½ ,, ,, ...	0·027	99·5

As at 37° C. so at 12° C. adsorption took place with great rapidity, over 90 °/o of the opsonin being removed in the first ten minutes. Further depletion occurred, however, at this temperature until no less than 99·5 °/o was adsorbed. The fact that at 37° C. equilibrium was reached at a very early stage, suggested that possibly under the influence of heat some dissociation of the adsorbed opsonin took place. The complete investigation of this question has not yet been attacked but it may be of interest to record the following experiment in which an attempt was made to demonstrate such dissociation as might occur.

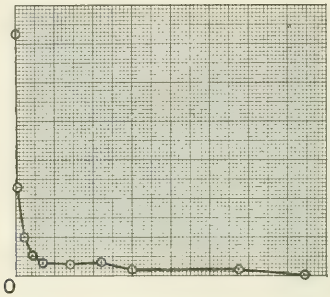

Chart XVI. Velocity of Adsorption. *x*. Time in minutes.
y. Phagocytic indices of supernatants.

Technique.

Three series of tubes were put up, each containing the same elements, viz.: 3 vols. end-piece + 1 vol. saline + 1 vol. bacillary emulsion.

Series A.

1. Immediately after mixing, the total volume was centrifugalised and the supernatant fluid collected.

2. Five minutes after mixing, the total volume was centrifugalised and the supernatant fluid collected.

3. Thirty minutes after mixing, the total volume was centrifugalised and the supernatant fluid collected.

As control the phagocytic index of the unadsorbed fluid was taken.

Series B.

1. Immediately after mixing, the total volume was centrifugalised. The clear supernatant fluid was then heated for 30 mins. at 56° C.

2. Five minutes after mixing, the total volume was centrifugalised and the supernatant fluid similarly treated.

3. Thirty minutes after mixing, the total volume was centrifugalised and the supernatant fluid similarly treated.

As control to this series, the phagocytic index of the *heated but unadsorbed* serum was taken.

Series C.

1. Immediately after mixing, the total volume was heated for 30 mins. at 56° C. and finally centrifugalised.
2. Five minutes after mixing, a similar procedure was carried out.
3. Thirty minutes after mixing, a similar procedure was carried out.

The control in this series was the same as that in Series B.

All the supernatant fluids in the three series were tested at the same time with the same fresh emulsion of *B. typhosus*.

Results: (see Chart XVII).

	Series A P. I.	Series B P. I.	Series C P. I.
Unadsorbed fluid	12·6	9·0	9·0
Immediately after mixing	3·4	2·3	1·8
5 mins. after mixing	1·3	0·72	1·08
30 mins. after mixing	0·65	0·39	0·83

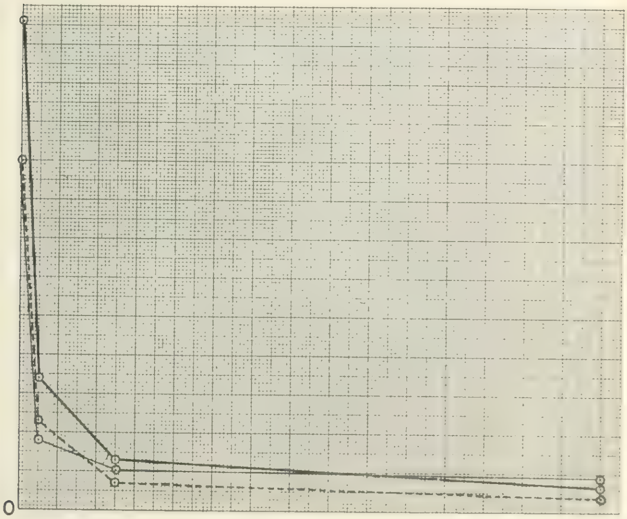

Chart XVII. ———— Series A. - - - - - - - - - - Series B. ———— Series C.

The series in which the supernatants were heated after centrifugalisation (Series B) show throughout slightly lower indices than Series A.

In Series C however (in which signs of dissociation were looked for) the indices after 5 mins. and 30 mins. are both appreciably higher than the corresponding indices in Series B which constitutes the proper control to Series C. The final index in Series C is, in fact, higher than the corresponding index in Series A in which no heat was applied either before or after centrifugalisation. I do not propose, however, to lay any stress on this result, as little more than a suggestion of dissociation of adsorbed opsonin by heat is indicated. The question will be again attacked by other methods.

STAGE III.

The phagocytosis of sensitised organisms suspended in salt solution.

In the experiments recorded in Stage II we have discussed the numerical relation subsisting between "bound" and "free" opsonin and we have shown that in equilibrium that relation can be expressed most accurately by an equation of the type characteristic of adsorption processes, viz., $\frac{y}{a} = Kx^n$, where "y" represents the amount of opsonin "bound," "x" the amount of "free" opsonin when equilibrium has been reached, "a" the concentration of bacilli employed for digestion and K and n are constants. The values of the exponent "n" in three carefully conducted experiments of this type have been respectively 0·158, 0·217 and 0·48. The same sensitising fluid (end-piece) was used throughout.

I now proceed to describe the technique employed in the experiments of Stage III which were devised with the object of determining by numerical methods whether the actual phagocytosis of sensitised bacilli removed from the serum used for sensitisation and resuspended in saline, could be explained as an adsorption phenomenon.

Several difficulties had to be contended with before a satisfactory technique could be evolved, but the main difficulty lay in the choice of an appropriate concentration of bacilli for the preliminary sensitisation process. In the earlier orientating experiments some of which will be recorded, the concentration of bacilli used for adsorption was much too great for the available opsonin. Consequently when these bacilli were freed from serum, suspended in saline and presented in various concentrations to the leucocytes, very low phagocytic indices were obtained. Under such conditions it was also observed that the leucocytes themselves underwent considerable lytic changes probably owing to the leucotoxic action of unneutralised bacterial extract. In some experiments (not recorded here) it was ascertained that leucocytes in the presence of saline extracts of partially sensitised bacilli frequently showed degenerative changes whereas during a similar period in the presence of saline only or a *heated* saline extract of partially sensitised bacilli, no obvious lytic changes were detected. Under this heading no difficulty was experienced when as in the later experiments emulsions in saline of fully sensitised bacilli were employed.

Experiment I. (Preliminary.)

One c.c. of end-piece was digested for 30 mins. at 37° C with 0·2 c.c. of a *thick* emulsion of *B. typhosus.* The supernatant fluid after centrifugalisation was removed, and the deposit of bacilli was washed twice with saline and again resuspended. Various dilutions of these bacilli in saline were then prepared.

Scheme of phagocytosis experiment:

1 vol. leucocytes + 1 vol. of bacillary emulsion.

Result:

Concentration of Bacilli	Phagocytic Index
1 in 1	$7{\cdot}2 = \frac{590}{81}$
1 in 2	$0{\cdot}7 = \frac{59}{83}$
1 in 4	$0{\cdot}6 = \frac{81}{127}$
1 in 8	$0{\cdot}5 = \frac{93}{185}$
1 in 16	$0{\cdot}4 = \frac{52}{117}$
1 in 32	$0{\cdot}07 = \frac{12}{170}$
1 in 64	?
1 in 128	$0{\cdot}07 = \frac{12}{168}$
1 in 256	$0{\cdot}06 = \frac{23}{337}$

A gradual rise in the phagocytic index as the concentration of bacilli increased is apparent, but the very low intake per leucocyte (except in the case of the top figure) suggested that the bacilli had been very incompletely sensitised.

Experiment II. (Preliminary.)

0·4 c.c. end-piece was digested for 50 mins. at 37° C. with 0·1 c.c. of a *thick* emulsion of bacilli.

Further technique as in Experiment I.

Result:

Concentration of Bacilli	Phagocytic Index
1 in 1	$1{\cdot}18 = \frac{242}{204}$
1 in 2	$1{\cdot}02 = \frac{210}{204}$
1 in 4	$0{\cdot}42 = \frac{86}{204}$
1 in 8	$0{\cdot}40 = \frac{77}{190}$
1 in 16	$0{\cdot}26 = \frac{54}{204}$
1 in 32	$0{\cdot}064 = \frac{23}{359}$
1 in 64	$0{\cdot}029 = \frac{10}{343}$
1 in 128	$0{\cdot}011 = \frac{4}{345}$
1 in 256	$0{\cdot}012 = \frac{5}{402}$
1 in 512	$0{\cdot}024 = \frac{8}{322}$

The indices are again low but there is a progressive increase in the intake per leucocyte as the concentration of bacilli increases.

Experiment III.

In this and later experiments, thin emulsions of *B. typhosus* were employed in the preliminary sensitisation process. By this means, a range of readily enumerable indices was obtained. Owing, however, to the resulting small deposit of sensitised bacilli, the range of indices was considerably shortened. In order therefore, to extend the range in the direction of the higher indices, recourse was had in some cases to the expedient of halving or quartering the leucocyte-concentration. In other cases, to attain the same object, several lots of bacilli were sensitised separately and the resulting deposits added together. It was then possible to obtain a satisfactory range of bacillary concentrations which would yield a satisfactory range of indices.

In Exp. III two lots of the following mixture were separately prepared and digested together for 20 mins.:

6 vols. end-piece + 2 vols. saline + 2 vols. thin emulsion of *B. typhosus*. The deposits were added together and washed twice in saline. Thereafter, various concentrations were put up.

Result:

Concentration of Bacilli	Phagocytic Index
4*	$18{\cdot}25 = \frac{1479}{81}$
2*	$22{\cdot}42 = \frac{1166}{52}$
1 in 1	$14{\cdot}92 = \frac{3552}{238}$
1 in 2	$8{\cdot}53 = \frac{1092}{128}$
1 in 4	$5{\cdot}33 = \frac{544}{102}$
1 in 8	$2{\cdot}70 = \frac{276}{102}$
1 in 16	$2{\cdot}01 = \frac{274}{136}$
1 in 32	$0{\cdot}65 = \frac{67}{102}$

* Obtained by dilution of the leucocytes.

Experiment IV.

The following mixture was digested for 10 mins.:

6 vols. end-piece + 2 vols. saline + 3 vols. bacillary emulsion.

The deposit of bacilli, after washing, was concentrated by reducing the volume of saline from 11 to 3. Various concentrations were then put up.

Result:

Concentration of Bacilli	Phagocytic Index
1 in 1*	$13{\cdot}29 = \frac{1356}{102}$
1 in 2	$12{\cdot}20 = \frac{1245}{102}$
1 in 4	$7{\cdot}20 = \frac{735}{102}$
1 in 8	$3{\cdot}55 = \frac{605}{170}$
1 in 16	$2{\cdot}16 = \frac{393}{181}$
1 in 32	$0{\cdot}73 = \frac{125}{170}$

* Obtained by dilution of the leucocytes.

The results of Exps. III and IV have not been submitted to mathematical analysis as the ratio B/L was not calculated and consequently the amount of bacilli "free" (*i.e.* not phagocytosed) could not be ascertained. In the following experiments this initial ratio was calculated.

Experiment V.

Result:

B/L	P. I. or Bacilli "Bound"	Bacilli "Free" (*i.e.* B/L – P. I.)	Percentage of Bacilli "Bound"
114	27·09	86·91	23·76
57	21·18	35·82	37·33
28·5	11·75	16·75	41·22
14·25	7·23	7·02	50·73
7·125	3·74	3·385	52·49
3·562	1·84	1·722	51·64
1·781	1·22	0·561	68·49
0·890	0·84	0·0506	94·31
0·445	0·36	0·0853	80·84

It will be seen that as the concentration of bacilli (and consequently the ratio B/L) diminishes, the percentage of "bound" or phagocytosed bacilli slowly but progressively increases.

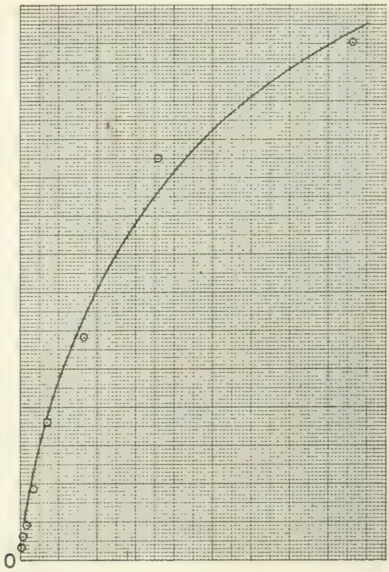

Chart XVIII. x. **Bacilli "free."** y. **Bacilli "bound."**

In Chart XVIII the bacilli "bound" (ordinates) have been plotted against the bacilli "free" (as abscissae), and in Chart XIX the logarithms of these quantities have been plotted. With the exception of the 8th which is badly out, all these points lie very closely on either side of a straight line. By the method of moments (omitting the 8th point) the value of 0·655 has been calculated for the exponent "n." This value yields the following figures for log K^1:

0·852	and for K^1 7·12
0·997	9·95
0·958	9·08
0·994	9·87
0·916	8·24
0·800	6·31
0·940	8·72
?	?
0·947	8·85

Considering the technical difficulties of the method, these figures must be regarded as highly satisfactory.

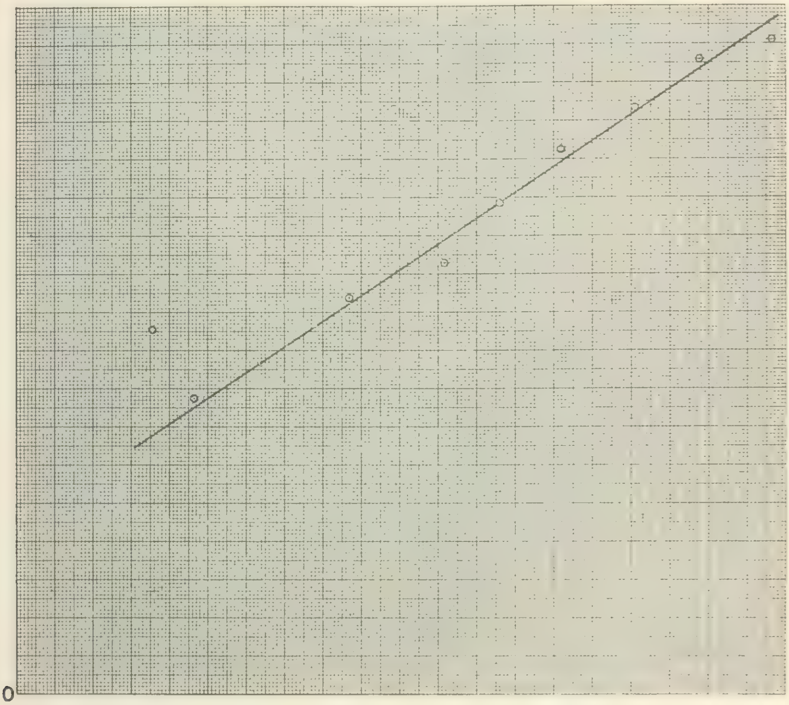

Chart XIX. x. Log. Bacilli "free." y. Log. Bacilli "bound."

Experiment VI.

Three lots of the following mixture were digested and the deposits collected and washed:

3 vols. end-piece + 1 vol. saline + 1 vol. bacillary emulsion.

The leucocyte and bacillary emulsions were counted so that the ratio of B/L in the various mixtures was calculated.

The rise in the percentage of bacilli "bound" as the initial concentration diminishes, is again very slow and gradual and the highest percentage reached is only 34·68 %.

The fall in the case of the two latter percentages is somewhat difficult to explain. It is most probably due to experimental error in estimating the final low indices.

Result:

B/L	P. I. or Bacilli "Bound"	Bacilli "Free" (*i.e.* B/L – P. I.)	Percentage of Bacilli "Bound"
536	29·22	506·78	5·4
268	17·35	250·65	6·4
134	16·20	117·8	12·08
67	9·47	57·53	14·13
33·5	5·86	27·64	17·49
16·75	4·02	12·73	24·00
8·375	2·19	6·18	26·16
4·187	1·45	2·73	34·68
2·09	0·46	1·63	22·0
1·04	0·18	0·86	17·22

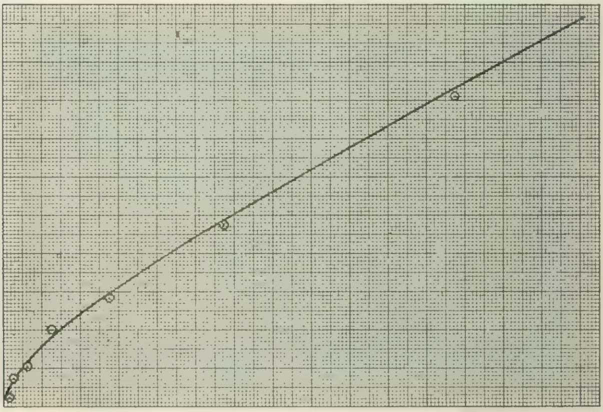

Chart XX. x. Bacilli "free." y. Bacilli "bound."

Analysis.

In Chart XX the "bound" bacilli are plotted as ordinates and the "free" bacilli as abscissae. In Chart XXI are plotted the points corresponding to the logarithms of these quantities. These points (with the exception of the 9th and 10th) are seen to lie closely on either side of a straight line.

By the method previously explained (omitting the two final points) a value of "n," viz. 0·577, was calculated which yielded the following values for log K^1:

0·354	and for K^1 2·26
0·301	2·00
0·464	2·91
0·403	2·52
0·376	2·37
0·404	2·53
0·319	2·08
0·342	2·19

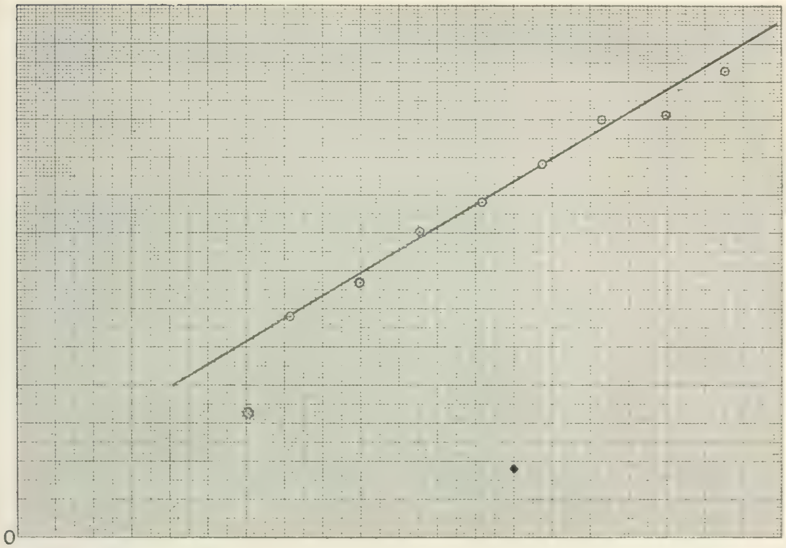

Chart XXI. x. Log. Bacilli "free." y. Log. Bacilli "bound."

In Chart XXII are plotted the amounts of bacilli "bound" as ordinates and of bacilli "free" as abscissae. In the same chart the logarithms of these amounts are plotted against each other. It is seen at a glance, that with the exception of the 7th all these points lie on a straight line.

Also a mere inspection of the figures for the phagocytic indices shows, that as the initial bacillary concentration is doubled, the phagocytic index is also very nearly doubled. Consequently the percentage of the bacilli "bound" remains almost constant. In reality, however, the percentages lie within a very short range from 22·8 % to 30·3 %. The value of the exponent "n" is thus very nearly unity.

Experiment VII.

Scheme: 6 vols. end-piece + 2 vols. saline + 2 vols. of thin bacillary emulsion digested together for 15 mins. at 12° C.

Deposit washed, resuspended in saline, and various concentrations prepared therefrom.

Result:

B/L	P. I. or Bacilli "Bound"	Bacilli "Free" (*i.e.* B/L − P. I.)	Percentage of Bacilli "Bound"
59	13·5	45·5	22·8
29·5	7·93	21·57	26·8
14·75	4·10	10·65	27·7
7·37	2·03	5·345	27·5
3·687	1·12	2·567	30·3
1·843	0·517	1·326	28·0
0·921	0·168	0·753	18·2

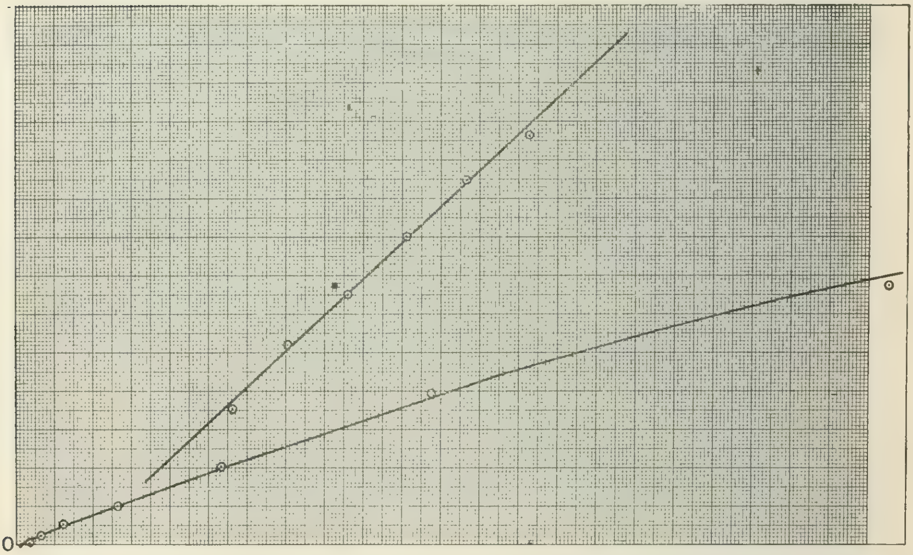

Chart XXII. x. Log. Bacilli "free." y. Log. Bacilli "bound." (Upper curve.)
x. Bacilli "free." y. Bacilli "bound." (Lower curve.)

By the usual method of calculation the value 0·92 for "n" gives the inclination of a straight line which passes most closely through the experimental points. The equation to this line which is drawn on the chart is $y = 0{\cdot}92x - 0{\cdot}277$.

General remarks on Experiments V, VI and VII.

In these three experiments we have endeavoured to ascertain by actual numerical methods whether the inclusion by leucocytes of sensitised bacilli suspended in a neutral fluid could be explained on an adsorption basis and we have shown that with due regard to difficulties of technique, the relation between the amounts of bacilli taken up by the leucocytes and the amounts left free in the surrounding fluid can be expressed most satisfactorily by an equation of the form $y = Kx^n$. The values obtained for "n" in these experiments, viz. 0·655, 0·577 and 0·92 are considerably higher than those calculated in the experiments of Stage II where the actual adsorption of opsonin by bacilli was discussed.

A further series of experiments similar to those of Stage III was contemplated but owing to exhaustion of the special sensitising fluid (guinea-pig end-piece) employed throughout the work, these experiments could not be carried out. It is proposed, however, to continue the investigation of this subject by aid of an end-piece derived from an immune antiserum obtained by immunisation of the rabbit with *B. typhosus* and the results recorded in the present communication though forming in themselves a fairly complete whole, are to be regarded in the light of a preliminary contribution to the subject.

SUMMARY AND CONCLUSIONS.

The two processes involved in the phagocytosis of bacilli (*B. typhosus*) by polymorphonuclear leucocytes in the presence of a sensitising fluid, viz. (1) the sensitisation of the micro-organism, and (2) the phagocytosis of the sensitised micro-organism, have been separately investigated. A single guinea-pig end-piece acted as the sensitising fluid throughout all the experiments.

The removal of opsonin by bacillary emulsions was found to run the course of an adsorption process as evidenced by the numerical relations found to subsist between the bound and free opsonin in equilibrium.

Also the actual phagocytosis of sensitised or partially sensitised micro-organisms suspended in a neutral fluid (physiological saline) was found, on analysis by similar numerical methods, to proceed as an adsorption process.

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A NOTE UPON THE USE OF ANTIFORMIN IN SPUTUM EXAMINATION,

AND ON STAINING METHODS FOR THE DEMONSTRATION OF TUBERCLE BACILLI.

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Since the routine examination of sputum as a means of diagnosis for tubercle became a universal practice, it has been recognized that diagnosis might be rendered more precise by some satisfactory method of rendering the sputum homogeneous, and dissolving the non-tuberculous elements therein contained. For this purpose such reagents as caustic alkalis, hydrogen peroxide, etc., were tried with a certain measure of success. The procedure of Ellermann and Erlandsen (1908), which consisted in autodigestion of the sputum in the presence of dilute sodium carbonate, and subsequent treatment with dilute sodium hydrate, was shown by careful workers (Jörgensen, 1910) to give results superior to those obtained with antiformin, but, nevertheless, the introduction of this latter reagent must be regarded as the chief recent advance in this branch of study. Antiformin is a proprietary substance which is made up of equal parts of caustic soda 15 per cent., and liquor sodae chlorinatae. It was first used in breweries for cleansing vats and for general disinfection. It is a very powerful solvent of organic substances, rapidly causing the disintegration and solution of such materials as pus, mucus, connective tissues, faeces, and hair. Waxes and waxy substances alone resist its action. Uhlenhuth and Xylander (1909) studied its solvent and its germicidal powers, and published the description of a method for its application to the investigation of tuberculous material. They found that, whereas other bacteria dissolved in the reagent as rapidly as sugar in water, tubercle bacilli retained their vitality in a 20 per cent. solution for twelve to twenty-four hours, and continued to exhibit their characteristic form and staining reactions for eight days.

Uhlenhuth made use of a 20 or 25 per cent. dilution of antiformin. This was added to sputum in equal bulk. The mixture was well shaken and incubated at 37° C. for six to twelve hours. Then after centrifugalization, the deposit was examined for tubercle bacilli in the ordinary way.

Lorenz (1911) used the same strength of antiformin, but heated the mixture over a flame, thus rendering the sputum more rapidly homogeneous. Haserodt (1909) shook up the sputum and antiformin mixture, after it had become homogeneous, with ligroin, and placed the mixture in a water bath at 60° C. The lighter fluid rising to the top, carried with it the tubercle bacilli, which collected at the zone of junction between the two layers of fluid. For this method no centrifuge is required.

When rapid concentration of tubercle bacilli in sputum is required, Hammerl's method (1909) is satisfactory. One part of sputum is shaken up with two parts of a 1 per cent. solution of caustic potash in strong ammonia. The mixture is incubated for half an hour, being shaken up from time to time. Then, after the addition of one part of acetone, to lower the specific gravity of the liquid, it is centrifugalized and the sediment examined. This method I have tried in a number of cases with good results. By this means, however, tubercle bacilli are rapidly killed, and therefore the method cannot be used for the purpose of obtaining pure cultures of tubercle. Antiformin, which does not kill tubercle bacilli if used in sufficient dilution, is well adapted for such cultural purposes.

In the routine work of the Lister Institute, the sample of sputum is mixed with an equal quantity of a 30 per cent. solution of antiformin, and the mixture is incubated overnight at 37° C. After centrifugalization, the supernatant fluid is poured away and replaced by an equal bulk of normal saline solution. This is shaken up with the deposit, and the mixture again centrifugalized. Films from the deposit thus washed adhere better to the slide than those prepared after one centrifugalization only.

The antiformin is diluted for use with distilled water. It was suggested by Beitzke (1910) that the use of tap water for this purpose might introduce a fallacy, owing to the occasional presence in such water of acid-fast bacilli. Organisms of this type have been found on water taps at the Lister Institute.

Results Obtained with the Antiformin Method.

Since March, 1911, all sputa received at the institute and found negative on direct examination have been treated with antiformin in the manner described above and further investigated. The number of examinations has been relatively large, and it has therefore been thought that statistics based upon these data would possess sufficient value to justify this note.

The number of sputa received for investigation during thirteen months has been 2,273. On direct examination 622 were found to be positive. The remaining 1,651 specimens, which gave negative results on direct examination, were treated with antiformin, and further investigation showed the presence of tubercle bacilli in 9 cases. The percentage of cases found positive on direct examination was thus 27.36, and the percentage of all positive cases 27.76. The cases found positive only after they had been treated with antiformin amounted to 0.4 per cent. of the total number of sputa investigated, and to 0.54 per cent. of those found negative on direct examination. The figure 0.54 may be described more concisely as the improvement percentage.

Comparison with Other Observations.

Other observers have obtained much higher percentages of improvement with the use of antiformin than that shown by our figures. Shortly after the introduction of the method, Meyer (1909) published a report upon the examination of 114 samples of sputum. Fifteen were found positive on direct examination. The remaining 99 samples were treated with antiformin, and further investigations revealed the presence of tubercle bacilli in 14 cases. Other contributions to the literature of this subject, too numerous to be cited in detail, give improvement percentages ranging from 10 to 35. Schneider (1912) has recently made a comparative study of some of the principal modifications of the antiformin method, and has obtained such improvement percentages as 17.5 and 22.8. He recommends the use of 15 per cent. antiformin, and that the examination be made as soon as possible after the material has become homogeneous. For the demonstration of bacilli, when very few are present, he considers the method of Lorenz the most satisfactory. Upon methods involving the use of ligroin and allied substances he reports unfavourably, being of opinion that such materials have a detrimental effect upon the staining powers of acid-fast bacilli.

In order to reconcile the results obtained at the Lister Institute with those quoted above, it is necessary to consider the procedure that is followed here. Care is taken in the first instance to select a suitable portion of the sputum for examination. The film prepared from this is examined, if need be, for four minutes. In the majority of cases tubercle bacilli, if present, are discovered within the first half-minute, but in a number of cases they are only detected after three or four minutes' search. Moreover, in two of the nine cases in which they were not found until after antiformin treatment, reference back to the original film showed the presence of one or two bacilli. The slides prepared from material which has been treated with antiformin are examined with no less care than the original film. Here bacilli, if present, were usually found in large numbers, but in one instance they were only discovered after a protracted search. In this connexion it is interesting to note the work of Meyer, who investigated the twenty-four hours' sputum of a number of sanatorium patients, and found in some cases such small numbers as two, four, or six bacilli.

No paper previously published upon the subject deals with such a large number of specimens as the present one, and the results of these observations appear to show that the greater the care expended upon the examination of the direct film, the less will be the apparent gain with the use of antiformin. This does not detract from the value of the antiformin method, as an improvement percentage as low as 0.54 involves an approximation to complete precision, which is of importance when the number of specimens examined is large.

SUMMARY.

By means of treating with 25 per cent. antiformin 1,651 samples of sputum found negative as a result of the direct examination, it was possible to demonstrate the presence of tubercle bacilli in 9 cases, that is to say, in 0.54 per cent. of cases. This figure, though small in comparison with those obtained by other workers, is large enough to justify the use of this reagent when a large number of specimens are examined. The discrepancy between these results and others is possibly due to the detailed care which is expended upon the direct examination.

STAINING METHODS.

A comparative study of the efficacy of certain staining methods for tubercle as applied to sputa has been carried out with a series of about 200 sputa. The methods dealt with have been those of Much, Herman, and Gasis.

Much's Method (1907) is a modification of Gram's method. Of the three different processes described by Much the following has been used. The fixed film is treated for a few minutes with hot carbol-methyl-violet solution (10 c.cm. of hot saturated alcoholic solution of methyl violet in 100 c.cm. of 2 per cent. carbolic acid). The slide is washed, and treated with Lugol's iodine for one minute, 5 per cent. hydrochloric acid for one minute, 3 per cent. hydrochloric acid for ten seconds, and a mixture of acetone and absolute alcohol in equal parts to complete the decolorization. One per cent. neutral red may be used as a counterstain.

Herman's Method (1908).—The stain, a 3 per cent. solution of crystal violet in 95 per cent. alcohol, and the mordant, 1 per cent. ammonium carbonate solution, are kept in separate bottles, and mixed for use in the proportion of 1 to 3. The fixed film is stained with this mixture over a small flame for a few minutes. The slide is washed, transferred to 10 per cent. nitric acid, and then to absolute alcohol. Herman does not use a counterstain, but in the present investigation a 0.3 per cent. chrysoidin solution has been found of service.

Gasis's Method (1907).—Corrosive sublimate crystals amounting to the size of a pea are boiled in about 5 c.cm. of a 1 per cent. eosin solution (eosin 1 gram, absolute alcohol 5 c.cm., distilled water 95 c.cm.). The hot stain is filtered on to the slide, and staining over the flame is allowed to proceed for two minutes. The slide is washed and then decolorized with alkaline potassium iodide solution (potassium iodide 1 gram, sodium hydrate 0.5 gram, 50 per cent. alcohol 100 c.cm.) and with absolute alcohol. Acid methylene blue is used as a counterstain.

Much claims that by the use of his method it is possible to demonstrate tubercle bacilli which are not shown in preparations stained by the Ziehl-Neelsen method. This granular non-acid-fast type of tubercle bacillus has been investigated by a number of workers, who have demonstrated its presence in tuberculous matter where no acid-fast bacilli of the ordinary type were to be found. Much also describes granules, visible in preparations stained by his method, but not seen in Ziehl-Neelsen slides, which are capable of causing tuberculous disease when inoculated into guinea-pigs.

Herman's method has been in use for a number of years, and its author claims that it is superior to the Ziehl-Neelsen method. He lays stress upon the advantage of using ammonium carbonate as a mordant, and states that in preparations stained by this process there are seen not only the bacilli which can be shown by the Ziehl-Neelsen method, but also the granules and granular bacilli described by Much.

Gasis's method differs essentially from the foregoing in that it is based upon the alkali-fast and not the acid-fast property of these organisms. It is stated that the alkalifastness is due to a nucleo-protein and not to a waxy component of the bacillus. The method was first introduced for the purpose of demonstrating tubercle bacilli in the urine, and the claim was made that in urinary sediments so stained, morphological characters distinguishing these organisms from smegma bacilli were rendered obvious.

Application of Staining Methods to Sputum Examination.

A series of 206 sputa were investigated by the methods of Ziehl-Neelsen and Herman. One hundred specimens were examined also by Much's method, and in 48 of the cases Gasis's process was also employed.

No. of Specimens.	Positive Results with			
	Ziehl-Neelsen.	Herman.	Much.	Gasis.
206	51	51	—	—
100	30	—	29	—
48	13	—	—	9

If the Ziehl-Neelsen figures be regarded as expressing 100 per cent. of accuracy, it is found that the other methods—Herman's, Much's and Gasis's—give respectively the figures 100, 96.66, and 69.23, indicative of their relative accuracy.

Gasis's method is the most unsatisfactory. The reagents are unstable, and require to be freshly prepared at the time of use, the staining process is troublesome and requires careful watching, and the results are uncertain, as the figures show. It possesses, however, considerable scientific interest, due to the peculiar biochemical reaction which underlies it. Bacilli stained by this means have a rose pink tint. The outline is well defined, and there is frequently to be seen some degree of granularity and of irregular vacuolation.

Much's method is not suitable for the direct examination of sputum, owing to the fact that a large number of Gram-positive cocci and bacilli other than tubercle take up the stain. This renders the process of search so troublesome that the routine use of the method for sputum diagnosis is not to be recommended. Tubercle bacilli so stained possess a highly granular character, and assume at times a streptococcal type, while free granules of the Much type are occasionally to be seen. The examination of the present series of films from sputum did not, however, afford any independent evidence of the existence of a type of tubercle bacillus demonstrable by this method which would not be shown more satisfactorily when stained according to Ziehl-Neelsen. The impression derived from a study of these specimens was rather that the marked granularity of tubercle bacilli on the Much-stained films was an artefact due to the nature of the staining reaction employed.

Herman's method is the only one of the three which can be compared with that of Ziehl-Neelsen for ease, utility, and efficiency. The necessity for keeping the solutions of ammonium carbonate and of crystal violet separate until they are required for use introduces a slight complication, which leaves the Ziehl-Neelsen method as the easiest, as it certainly remains the most efficient, process at our command. It is largely a matter of individual taste whether it is more desirable to search for violet bacilli upon a yellow ground, as with slides stained by Herman's method, or for red bacilli on a blue ground, as with Ziehl-Neelsen; but when the eye has become trained to one method, it requires to be educated afresh before equally satisfactory results are obtained with slides stained otherwise. Bacilli stained according to Herman present interesting morphological characters. Their outline is often irregular and granularity is well marked. Metachromatic spore-like granules were frequently noted, and branching forms were observed on two occasions.

An interesting account of various methods of staining tubercle bacilli, describing the earliest processes, which possess now a purely historical interest, and giving a comparative study of the efficiency of the recent methods, has recently been published by Böhm (1912). This author considers the method of Gasis interesting but unreliable, and finds the Ziehl-Neelsen method superior to all others.

The results detailed above show that the Ziehl-Neelsen method, which has stood the test of time, remains the most suitable of all for the detection of tubercle bacilli in urine. Next to it comes the method of Herman, almost as simple, quite as easy of execution, and giving equally good results. Much's method is not suitable for direct investigation of sputum, and the method of Gasis is both difficult and unreliable.

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LXIII. THE PREPARATION OF ANTITOXIC PLAGUE SERA.

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That it is possible to obtain an antitoxic as distinguished from an anti-infectious plague serum was shown by Markl (1898, 1901, 1903) and other workers. The strongest serums have been those obtained by Dean (1906) and Rowland (1911). The former used for the immunisation of horses the filtrates from old (8—10 weeks) bouillon cultures and obtained a serum of which 1 c.cm. neutralised either 150 or 450 M.L.D. of the toxin according as one takes the death-time-limit of the M.L.D. to be 48 hours or 4 days. Rowland used a toxin extracted from the plague bacillus by means of his "sulphate process" and the serum so obtained was found to neutralise about 100 M.L.D. per c.cm.—the death-time-limit of the M.L.D. being 48 hours. In order to compare these two serums we must base our calculations on the 48 hour limit, and judging by this standard there is not much to choose between them. This 48 hour death-time-limit is the most convenient one to take as the *majority* of rats which have received an injection of plague toxin either die within 48 hours or live longer than 7 days. For this reason it was decided to take this limit in the following experiments and *for the purposes of comparison* to consider any animal alive on the 3rd day as cured of the intoxication due to the plague toxin. For therapeutic purposes a serum which kept animals alive for 3 days only would of course be useless, but experiments made with such a serum might well point out the road to the attainment of better results.

It seems advisable to call attention here to the fact that occasionally many rats which survived the acute attack and which had at the end of a week apparently quite recovered from the inoculation of the toxin-serum mixture, died later in a condition of marasmus. This may be due either to the failure on the part of the serum to neutralise some constituent of the toxin which comes into play after the acute attack has passed off or, as some results suggest at first sight, to the serum itself possessing toxic properties. For example, the following mixtures of toxin and antitoxin were made (the volume in each case being made up to 4 c.c.), allowed to stand at room temperature for $\frac{3}{4}$ hour and then injected into rats subcutaneously.

Calcutta (bleeding 3. 2. 12). Rats 90—95 grms.

2·1 mgm. toxin + 1 c.cm. serum = † within 48 hours
,, ,, ,, + 0·75 ,, = lived
,, ,, ,, + 0·5 ,, = † by morning of 5th day
,, ,, ,, + 0·25 ,, = lived
,, ,, ,, + 0·1 ,, = lived
,, ,, ,, + 0·04 ,, = lived
,, ,, ,, + 0·02 ,, = † within 48 hours
,, ,, ,, + 0·01 ,, = † ,, ,,

† = died

Now the M.L.D. of this toxin was found to be between 0·56 mgm. and 0·7 mgm. and 2·1 mgm. was considered to be equal to three available minimal lethal doses. We are then faced with the paradox that while 1 c.cm. of serum failed to neutralise 3 M.L.D. this amount of toxin was rendered ineffective by 0·1 c.cm. and 0·04 c.cm.

If this result be due to some toxic property inherent in the serum itself, then large doses of serum alone ought to have a similar effect. But this is not the case as I have injected subcutaneously into rats 10 c.cm. of an antiplague serum, which gave this "paradox-result," without any ill effects. On another occasion 2 c.cm. of three other similar sera were injected subcutaneously into three rats. Two of these rats seemed ill on the second day, but they soon became quite well again. So it cannot be said that the serum itself is toxic in the doses given.

Besides what I have for convenience termed the "paradox-result," there is another source of confusion in evaluating a serum. It has been shown by Rowland (1910, p. 544) that a certain dose of his virulent plague culture will when injected subcutaneously into rats kill on an average only 70 out of 100, *i.e.* with regard to this particular dose some 30% of rats are immune. As there is this natural immunity to infection, it is allowable to presume that the opposite condition may also occur, and that some rats may be more markedly susceptible than others to the influence of plague toxin. It is to these causes that are due the irregularities which not infrequently appear in the tests.

The result of the following experiment may be taken as showing the existence of an increased susceptibility,

0·553 mgm. toxin + 4 c.cm. normal rat serum = lived
,, ,, ,, + 3 ,, ,, ,, = died
,, ,, ,, + 2 ,, ,, ,, = lived
,, ,, ,, + 1 ,, ,, ,, = ,,

while the opposite condition, namely one of immunity, appears to be present in the case of the first rat in the series below:

1·66 mgm. toxin + 1 c.cm. normal rat serum = lived
0·88 ,, ,, + ,, ,, ,, ,, = died
0·76 ,, ,, + ,, ,, ,, ,, = ,,

A good example of irregularities due to the causes above mentioned is furnished by the tests of a sample of serum from the horse *Lemberg*.

Lemberg. 10. 4. 12.

1*st test* 11. 4. 12. Rats weighing 95—110 grm. Inoculations subcutaneous.

2·1 mgm. toxin (3—4 M.L.D.'s) + 1/60 c.cm. serum = lived
,, ,, + 1/80 ,, = †within 24 hours
,, ,, + 1/100 ,, = lived
,, ,, + 1/150 ,, = †within 36 hours

The animals that lived did not show any symptoms of illness and so it was concluded that a repetition of this test would show definitely what was the value of this serum.

2*nd test* 15. 4. 12. Rats weighing 90—100 grm. Inoculations subcutaneous.

2·1 mgm. toxin + 1/60 c.cm. serum = †within 24 hours
,, + 1/80 ,, = † ,,
,, + 1/100 ,, = † ,,
,, + 1/150 ,, = † ,,

It is obvious that this second test does not help us. We can only infer either that the rats in the second test were hypersusceptible or that two of the rats in the first test were immune. So it was necessary to test this serum a third time.

3*rd test* 18. 4. 12. Rats weighing 90—120 grm. Inoculations subcutaneous.

2·1 mgm. toxin + 1/20 c.cm. serum = lived
,, + 1/30 ,, = ,,
,, + 1/40 ,, = ,,
,, + 1/50 ,, = ,,
,, + 1/60 ,, = †within 36 hours

We may now conclude that the antitoxic value of this serum is represented in the above test by $\frac{1}{50}$ c.cm. and that consequently 1 c.cm. = 150 M.L.D.

An endeavour was made to obtain in the case of each sample tested a clean result such as that given above, but it was not possible to attain this end in every case. In those cases where a result was obtained similar to that of the first of the above tests the serum would be valued at $\frac{1}{60}$th c.cm. or 180 M.L.D. per c.cm.; when the result was like that of the second test one could only say that the serum did not neutralise 180 M.L.D.'s per c.cm.

The next point to be considered is the toxin used as a test toxin. I have used various products as follows:

(1) *filtrates* of broth culture are rarely toxic enough and are highly unstable. Thus in 29 trials, only twice was a filtrate obtained of which 0·1 c.cm. would kill a rat; in one instance a filtrate, which, when first tested, killed a mouse of 25 grammes within 24 hours in a dose of $\frac{1}{100}$th c.cm. subcutaneously, had no effect three days later on a mouse of the same weight in a dose of $\frac{1}{15}$th c.cm.

(2) *Solutions of the toxic nucleoproteid* give good results when used fresh, but they lose toxicity steadily (vol. XI. supplement, p. 35); ultimately they seem to become relatively stable, but the results then obtained with them are quite different from those obtained with the same serum against fresh toxin solution, and call to mind the suggestion that there may be more than one toxic substance in the plague bacillus.

(3) *A dry preparation of the toxic nucleoproteid* has also been tried but gave irregular results in the serum tests.

(4) The most constant preparation which I have tried has been obtained by taking a quantity of the dry mass formed by mixing the bacilli with anhydrous sodium sulphate, melting it by placing it in the incubator at 37° C., obtaining an even distribution of the bacilli by thorough stirring and shaking and then solidifying the mixture quickly by pouring it into a shallow basin cooled by ice. This "toxin rock" (TRk 1) may be broken up into small pieces and kept in a stoppered bottle in the dark. When required for use, a small quantity is weighed out, dissolved in 0·85 % NaCl at 36° C., cooled to about 0° C. and filtered cold[1].

In the experiments detailed below

"*fresh toxin*" = a freshly prepared solution of toxic nucleoproteid,
"*SR* 2" = an old autolysed solution of the same,
"*SRNP* 1" = the toxic nucleoproteid in the form of a dry powder,
TRk 1" = the "rock" of sulphated bacilli.

In the following table are given the results of a series of tests of the same serum with various toxins.

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[1] A second sample of this "rock" did not give such satisfactorily constant results.

Serum Y 83. *Wealdstone* bleeding 28. 1. 11.

Date when tested	Toxin used as test toxin	No. of rat minimal lethal doses neutralized per c.cm. of serum
13. 4. 11	Bouillon filtrate 7 c.	500
28. 6. 11	SR 2	50
1. 7. 11	Fresh toxin	50 ? +
4. 7. 11	SR 2	30
13. 10. 11	,,	< 2
16. 10. 11	Fresh toxin	160
28. 11. 11	SR NP 1	? 10 ? 0
1. 12. 11	,,	? 20 ? 0
,,	Fresh toxin	600
30. 1. 12	TRk 1	60 +
1. 2. 12	,,	150

With the idea of bringing out the differences more plainly the details of one or two of these tests are given below.

Serum Y 83. Tested 1. 12. 11. Toxin SR NP 1. Rats weighing 90—105 grms.

Serum. 3 mgm. toxin + 1 c.cm. serum = † within 18 hours
,, + 0·5 ,, = † ,, 48 ,,
,, + 0·25 ,, = † ,, 48 ,,
,, + 0·1 ,, = † ,, 6th day
,, + 0·05 ,, = lived

Controls. 0·75 mgm. toxin = lived
0·5 ,, ,, = † within 48 hours

Note. The M. L. D. of this toxin usually lay between 0·75 mgm. and 0·5 mgm.

Serum Y 83. Tested 1. 12. 11. Toxin = fresh toxin. Rats weighing 85—120 grms.

Serum. 3 mgm. toxin + 1 c.cm. serum = † within 18 hours
,, + 0·5 ,, = lived
,, + 0·25 ,, = † 5th day
,, + 0·1 ,, = lived
,, + 0·05 ,, = † within 18 hours

Controls. 0·75 mgm. toxin = † within 18 hours
0·5 ,, = † ,, ,,
0·25 ,, = † ,, ,,
0·1 ,, = † 48—60 hours
0·05 ,, = † within 48 hours

Inoculations in each case were made subcutaneously. Toxin and serum in contact for $\frac{3}{4}$ hour at room temperature before injection.

The tests of this serum performed on 30. 1. 12 and on 1. 2. 12 using as test toxin "toxin rock" TRk 1 were of quite a different order:—

30. 1. 12. Rats weighing 90 grms.

2·1 mgm. toxin + 1 c.cm. serum Y 83 = no symptoms, lived
,, + 0·5 ,, ,, = ,, ,,
,, + 0·25 ,, ,, = ,, ,,
,, + 0·1 ,, ,, = ,, ,,
,, + 0·05 ,, ,, = ,, ,,

1. 2. 12. Rats weighing 90—115 grms.

2·1 mgm. toxin + 0·05 c.cm. serum = no symptoms, lived
,, + 0·02 ,, = ill, lived
,, + 0·01 ,, = † within 48 hours
,, + 0·005 ,, = † ,, ,,

NOTE. The M.L.D. of toxin TRk 1 lay between 0·7 mgm. and 0·56 mgm. and nearer the latter than the former.

It is clear that toxin rock TRk 1 gave the cleanest results and as it acted similarly with other serums it was taken into use as a test toxin. We see also from the tests of Y 83 that neither the autolysed toxin SR 2 nor the toxic nucleoprotein powder SRNP 1 are suitable to be used as test toxins. That filtrates of bouillon cultures, which are in reality solutions of autolysed toxin, should give better results than a toxin such as SR 2 is peculiar, but is interesting when considered in connection with Kolle's statement (1903, p. 361) that neither Bern nor Paris serum nor the serum produced by the injection of centrifuged old bouillon cultures had any neutralising effect upon the toxin contained in old bouillon cultures, and that he could not confirm Markl's statement that it is possible to produce an antitoxin against the poison of old cultures. This behaviour of toxin SR 2 is very puzzling and recalls the suggestion that there may be two toxins produced by the plague bacillus, and that this may be the same kind of toxin as is spoken of by Kolle. Or Palandino-Blandini's (1905) statement may apply to toxin as well as cultures. He says that typhoid and plague serum protect against higher multiples of the M.L.D. when virulent strains are used than when weakened strains are used, because in the last case a larger comparative amount of bacterio-nuclein is injected and the bacterio-nuclein influences the protective power of the serum. This can only apply if there is a total destruction of toxin. But if we consider the test made on 4. 7. 11 another possible explanation is forthcoming. The details of that test are as follows:

Y 83 serum. Toxin SR 2. Rats weighing 90—95 grms.

3 c.c. toxin SR 2 + 1·0 c.c. serum = no symptoms, lived
,, ,, + 0·5 ,, = ,, ,,
,, ,, + 0·25 ,, = ill but recovered
,, ,, + 0·1 ,, = ,, ,,
,, ,, + 0·05 ,, = † within 24 hours

Serum and toxin in contact for ¾ hour before injection.

This is a perfectly clean result and we can say definitely that 0·1 c.cm. of serum neutralized 3 c.cm. of toxin. Now at the time of the test the M.L.D. of this toxin was 0·75 c.cm. or a little less, but not

0·5 c.cm., and the test dose would contain 4 M.L.D. of which we can only count three as available for evaluating the serum, so that 1 c.cm. of serum would neutralise 30 M.L.D. But the original M.L.D. of this toxin was about 0·2 c.cm. which would make the test dose of 3 c.cm. equal to 15 M.L.D., and as this was neutralised by 0·1 c.cm. of serum the serum would be per c.cm. equivalent to 150 original M.L.D. This titre is the same as the serum had when tested against toxin TRk 1 which was an unmodified toxin.

This is rather against the existence of two separate toxins and also against an absolute destruction of toxin and suggests that, as in the case of diphtheria toxin, the plague toxin has become modified into "toxoid"—some ¾ of each original M.L.D. having been thus converted.

Such a toxin, *i.e.* one that has become modified by being allowed to undergo autolysis, is capable of producing immunity and antitoxin as is shown by the case of the horse *Bombardier* which was immunised with comparatively non-toxic toxin. When first prepared this toxin had an M.L.D. for rats of about 0·2 mgm., but after being kept for 4–6 weeks at 36° C. the M.L.D. had risen to about 3 mgm. The details of the immunisation are given below. The toxin was injected into the muscles of the neck just in front of the scapula.

Immunisation of Bombardier.

Date	Mgms. of toxin inoculated	No. of rat M. L. D.'s inoculated	Sample bleeding	Date of test	Toxin used as test	No. of rat M. L. D. neutralised per c.cm
May 4, 1911	10	3–4	—	—	—	—
,, 16 ,,	20	7	—	—	—	—
,, 26 ,,	40	14	—	—	—	—
June 9 ,,	80	28	—	—		—
July 4 ,,	150	50	—	—		—
,, 13 ,,	300	100	—	—	—	—
,, 14 ,,	—	—	20 c.cm.	—	—	—
,, 20 ,,	—	—	,,	20. 7. 11	SR 2	5
,, 27 ,,	600	200	—	—	—	—
,, 28 ,,	—	—	20 c.cm.	28. 7. 11	SR 2	5
,, 30 ,,		—	,,	31. 7. 11	,,	5
Aug. 1 ,,	—	—	,,	1. 8. 11	,,	5
,, 3 ,,	-	—	,,	4. 8. 11	,,	5
,, 4 ,,	520	170	—	—	—	—
,, 9 ,,	1000	333	—	—	—	—
,, 16 ,,	—	—	20 c.cm.	16. 8. 11	SR 2	30

From a study of this table we learn several things. Firstly we see that injections of this modified toxin can give rise to the production of antitoxin. The amount of antitoxin was at first quite as large and finally much larger than that usually found in the serum of horses immunised according to Yersin's method, *i.e.* intravenous inoculations of living cultures.

Secondly, we see that the introduction of 600 mgm. in divided doses produced an antitoxin content which was not increased when 600 mgm. was given in one dose after an interval of 14 days, but that 1520 mgm. given in two doses with only a five-day interval increased the amount of antitoxin in the blood six times.

Lastly we see that the quantity of antitoxin in the blood was the same 24 hours after as it was before inoculation, and was found to be still at the same level when tested on the third, fifth and seventh day after injection. If this modified toxin caused a "negative phase" one would expect to find evidence of it 24 hours after the inoculation of such a dose as 600 mgm. (the growth from 171 Roux bottles). We may conclude that there is in this case either no negative phase or one with a duration of less than 24 hours. Now if such a large dose causes only a short negative phase or none at all, it is justifiable to presume that a small dose, such as would be used as a prophylactic measure, would not have any ill effect and consequently that such a modified toxin would be eminently suitable for use as a "vaccine."

It will have been noticed that during the immunisation of *Bombardier* samples of blood were taken at intervals of 24 hours, 3 days, 5 days and 7 days after the injection of 600 mgm. of modified toxin, and that the antitoxin content of the serum was the same on each occasion. The question then naturally arises "Does this condition obtain after doses of other toxins and at what time after inoculation does the serum contain the greatest quantity of antitoxin? When horses are immunised by means of intravenous injections of living cultures, it is usual to bleed about three weeks after the last injection, but the reasons for choosing such a late date in the case of living cultures need not necessarily hold good when toxins are used. So a series of samples of blood were taken at intervals of 7, 14 and 21 days after an injection and the serum tested for antitoxin. The results are given in the following table.

From a consideration of the table one would draw the conclusion that the antitoxin content of the blood does not vary much between the 6th and the 21st day. This condition proved most convenient as it enabled one to space the doses nicely and at the same time to take samples to ascertain the effect of each dose—thus a sample could be taken and then a dose given at weekly intervals.

Name of Horse	No. of rat M.L.D. neutralised per c.cm. of serum			Remarks
	7 days	14 days	21 days	
Lemberg	100 *	<120 †	<120 †	* Tested against bouillon filtrate 7 c. † ,, ,, fresh toxin
,,	9	9	9	Tested against toxin SR 2
Whiteface	30	30	30	,, ,, ,,
Calcutta	30	30	30	,, ,, ,,
,,	30	? 30	30	,, ,, ,,
,,	75	75	—	,, ,, TRk 1
,,	750	525 (12 days)	—	,, ,, ,,
Wealdstone	30 (6 days)	? 30 (13 days)	60 (20 days)	,, ,, SR 2
,,	75	50	—	,, ,, TRk 1
,,	225	240 (12 days)	—	,, ,, ,,

Proceeding in this manner with three horses which had already been immunised the results embodied in the following table were obtained:

Horse	No. of rat M.L.D. neutralised per c.cm. of serum					
	Before immunisation	7 days after 10 mgm.	7 days after 25 mgm.	7 days after 50 mgm.	7 days after 75 mgm.	14 days after 75 mgm.
Wealdstone	12	12	30	75	75	50
Calcutta	60	60	12	30	75	75
Lemberg	30	30	30	30	75	75

NOTE. The toxin used for immunisation was freshly prepared before each injection and the size of the dose is given in milligrammes of nucleoprotein as calculated from an analysis of a similar powder of "sulphated bacilli." The doses must be considered not as absolutely accurate but only approximate, for there are always slight differences in the nucleoprotein content of different batches of powder.

It appears that in the case of an immunised horse, which is resting after a bleeding, one inoculation of freshly prepared toxin has not much effect upon the antitoxin content of the blood—as obtains also in the case of diphtheria and possibly of other toxins. The effect of a 2nd, 3rd and 4th dose is somewhat variable when each dose is considered separately, but the summation effect is to stimulate the production of antitoxin.

The amount of antitoxin production in these three horses in consequence of the injection of 112 mgm. of fresh toxin was not great, and so they were given a rest and then inoculated with an autolysed toxin. From the experience gained with *Bombardier*, it was decided to try the effect of giving the doses at intervals of three days instead of a week but as the reaction following the second dose had not passed off by the third day, the third dose was given seven days after the second, and a sample taken to ascertain the effect of the first two doses.

Samples were also taken at various intervals after the third dose. The antitoxin content of these serums is given in tabular form below.

	No. of rat M.L.D. neutralised per c.cm. of serum								
Horse	8 days before inoculation	7 days after 100 and 200 mgm.	7 days after 300 mgm.	12 days after 300 mgm.	19 days after 300 mgm.	24 days after 300 mgm.	32 days after 300 mgm.	30 days after 300 mgm.	46 days after 300 mgm.
Wealdstone	50	150	225	240	300	—	300	90	90
Calcutta	75	300	750	525	360	450	300	300	120
Lemberg	75	210	300	300	450	—	240	150	150

Note. In the case of Lemberg owing to scarcity of toxin the third dose was only 100 mgm. instead of 300 mgm. as in the case of the other two horses.

We see that, just as in the case of *Bombardier*, doses of autolysed toxin at short intervals have produced a marked rise in the antitoxin content of the serum. The horses were bled 8 litres on the 12th day after the last injection, and in each case a slight improvement in the serum took place 7–12 days after bleeding. After that a gradual decrease occurred in the amount of antitoxin in the blood, until on the 46th day after the last injection the strength of the serum was only about double what it was before this series of injections was begun. On this date re-immunisation was commenced with a solution of toxin which had been kept at 36° C. for 1 month. This solution was made up to contain 1 mgm. of nucleoprotein per c.cm., and when fresh the M.L.D. for rats was about 0·7 mgm. After a month at 30° C. the M.L.D. had increased to 3 mgm. or 4 times as much as it was originally. The doses given were 30 mgm., 100 mgm. and 300 mgm., corresponding respectively to about 43, 148 and 430 original M.L.D. or 10, 33 and 100 M.L.D. of the autolysed toxin. The results were

	No. of rat M.L.D. neutralised per c.cm. of serum			
Horse	Before re-immunisation	3 days after 30 mgm.	7 days after 100 mgm.	9 days after 300 mgm.
Wealdstone	90	150	300	240
Calcutta	120	180	300	300
Lemberg	150	150	<180	240
Kitasato	60	60	60	90

Here again we see that the response to autolysed toxin is better than to fresh toxin. Of course it may be said that the doses of fresh toxin were not so large nor given in such a short time as those of the autolysed toxin, and that therefore the results are not comparable. Such criticism is quite fair and one would have liked to have avoided it, but from my experience I should say that such doses of fresh toxin could not be injected at such short intervals because the reaction following inoculation would be too great to allow of its being done. But we may compare the toxins not according to volume in c.cms. or weight in mgms., but according to the number of M.L.D. given. If we value the toxins according to their M.L.D. at the time of inoculation there is not so very much difference between the doses. In the first series the total number of M.L.D. was about 160, in the second about 200 and in the third about 144 M.L.D. If however we value them according to the M.L.D. when freshly prepared the differences are great, and the respective numbers would be about 160, 850 and 600 M.L.D. The difference in the amounts of antigen given in the above three series of inoculations may possibly be made more striking if we calculate the quantity of growth which was required to produce the nucleoprotein. Rowland's analyses have shown that on an average the growth from 1 Roux bottle yields 3·5 mgm. of nucleoprotein, and taking this amount as the basis of our calculations we find that the number of Roux bottles used was

Horse	1st series	2nd series	3rd series
Wealdstone	47	198	123
Calcutta	47	198	123
Lemberg	47	132	123

and the results suggest that other things being equal the response to an inoculation is more dependent on the amount than on the absolute toxicity of the antigen introduced. This agrees with the opinion expressed by Kretz (1909, p. 26), that the effect of the toxin in the production of antitoxin (diphtheria) depends not so much on its direct toxic value as on its antitoxin neutralising power—old weakened toxin giving as good results as fresh toxin.

Besides the toxicity and amount of antigen given there is another factor which influences the result. As in the cases of diphtheria and of tetanus so in the case of plague, each horse does not respond alike to a given stimulus. This is well illustrated by the results of the immunisations of the two horses *Ramsay* and *Wealdstone.*

Ramsay had between the 20th July 1910, and the 19th October 1910, 1340 mgm. of *fresh* toxin equal to about 4000 rat M.L.D. or the growth from some 380 Roux bottles, and yielded a serum of which 1 c.cm. neutralised some 400 rat M.L.D. *Wealdstone* had the first injection on Aug. 24, 1910, and by Oct. 10 had received 200 mgm. of fresh toxin. On Oct. 19th the serum had a titre of 150 M.L.D. per c.cm. The immunisation was continued, and by Jan. 7, 1911, the total quantity of toxin introduced was 3000 mgm. or 9000 rat M.L.D. or the growth from some 860 Roux bottles. On Jan. 28, 1911, the titre of the serum was per c.cm. 500 M.L.D. when tested against a toxic bouillon filtrate, but only some 150 M.L.D. when tested against "fresh toxin." For our present purpose we will take the larger of these two values and comparing it with that of *Ramsay* we see that doubling the amount of antigen has only raised the titre of the serum by a fraction and that therefore the "individuality" of the horse has a great deal to do with the response which follows the introduction of the plague antigen. *Wealdstone* had still larger doses of toxin—1000 mgm. 2000 mgm., 2000 mgm. and 3000 mgm. in single doses without any resulting increase in the titre of the serum.

We came therefore to the conclusion that it was useless to increase the size of the doses of fresh toxin to any great extent, not only because they seemed to be ineffectual in increasing the antitoxin content, but also because the local and constitutional reactions were so great that the intervals between the inoculations had to be lengthened from the 4 or 5 days usual at the commencement to one month at the end.

Both fresh and old toxin, but particularly the former, when given in doses of 100 mgm. and upwards cause a loss of flesh which is not to be accounted for by the temporary loss of appetite and the febrile condition brought about by the injection.

To sum up we may say that in the case of horses which have been already immunised, old weakened toxin appears to stimulate the production of antitoxin better than fresh toxin, and it has the great advantage of not interfering so much with the well-being of the animal, cf. Pane, 1911, p. 279.

In these tests the toxin and serum have been mixed and allowed to remain in contact for about $\frac{3}{4}$ hour at room temperature before injection into the rats. Other workers however have used a higher temperature and allowed a shorter or a longer time for union to take place, and so experiments were made to ascertain the effect of these two factors. Toxin and serum were mixed, the volume when necessary made up to about 4 c.cm., and the mixture allowed to stand at 36° C. for $\frac{3}{4}$ hour and for $1\frac{1}{2}$ hours. Control doses of toxin alone were also diluted and kept at 36° C. for the same time. There was no difference in the results following the injection of mixtures or pure toxin kept at room temperature or at 36° C. for $\frac{3}{4}$ hour or for $1\frac{1}{2}$ hours.

The next point considered was whether other antisera possessed the power of neutralising plague toxin to any great extent. For this purpose the following tests were performed.

Various antisera.

1 c.cm. of serum was mixed with decreasing amounts of plague toxin, made up to about 4 c.cm. when necessary, allowed to remain at room temperature for ¾ hour and injected subcutaneously into rats. The M.L.D. of the toxin used was between 0·7 mgm. and 0·56 mgm. The numbers show the weights of the rats in grammes.

Name of horse	Variety of serum	Date of bleeding	Date of experiment	Amounts of toxin			
				2·1 mgm.	1·4 mgm.	0·7 mgm.	0·56 mgm.
Tasmania *Melbourne* *Adelaide* *Hobart* *Wellington*	Pooled serum of 5 normal horses	28. 6. 11 29. 6. 11 29. 6. 11 29. 6. 11 29. 6. 11	12. 2. 12	+ 90	+120	+120	lived 120
Bangor	Antitetanic serum	9. 1. 12	12. 2. 12	+ 90	+ 90	+ 90	+ 90
Vignette	Had had injections of living streptococci and also of diphtheria toxin	19. 1. 12	12. 2. 12	+ 90	+ 95	lived 90	lived 90
Maude	Antistreptococcic serum	1. 11. 11	12. 2. 12	+ 90	+ 90	lived 90	lived 95
Arkwright	Anti pseudotubercle serum	10. 6. 8	19. 2. 12	+100	+ 90	+ 90	lived 90
Broncho	Antidysentery (intravenous inoculations)	26. 7. 6	28. 2. 12	+105	+110	+100	+100
Nobby	Antidysentery (intramuscular inoculations)	28. 2. 12	4. 4. 12	+105	lived 120	lived 120	lived 115
,,	Antidysentery (intramuscular inoculations)	29. 3. 12	4. 4. 12	+120	lived 125	lived 120	lived 120
Rich	Antistaphylococcic	21. 2. 07	28. 2. 12	+ 90	+100	+100	lived 105
Whitestreak	Antidiphtheritic (1300 units)	8. 2. 12	28. 2. 12	+115	+115	+100	lived 105
White Lady	,, (150 units)	12. 1. 11	28. 2. 12	+120	+120	lived 120	+120

Antiplague serum.

2·1 mgm. of plague toxin were mixed with decreasing amounts of antiplague serum, made up to 4 c.cm. where necessary, allowed to remain in contact ¾ hour at room temperature and then injected into rats subcutaneously.

Name of horse	Date of bleeding	Date of test	Amounts of serum					
			1 c.cm.	0·5	0·25	0·1	0·02	0·01
**Corbally*	22. 1. 09	28. 2. 12	lived 90	lived 100	lived 110	† 110	—	—
**Whiteface*	15. 2. 09	23. 2. 12	,,	lived 95	† 90	† 90	—	—
† ,,	26. 2. 12	26. 2. 12	,,	lived 90	lived 90	lived 90	—	—
Ramsay	5. 11. 10	28. 2. 12	—	—	—	—	lived 115	lived 90
Wealdstone	14. 3. 12	20. 3. 12	—	—	—	—	lived 90	† 100

* Immunised with living cultures given intravenously.

† ,, ,, filtrates intramuscularly.

From these results we are justified in concluding that neither normal serum nor any of the ordinary antisera are capable of neutralising plague toxin to any appreciable extent compared with anti-plague serum.

Having determined that the antitoxin in antiplague serum is a specific antitoxin two questions naturally occur to one. Does it retain its antitoxic power for any length of time unimpaired? What effect will tropical temperatures have upon it?

As regards these questions Chouroupoff (1909) examined the protective and curative properties of ordinary antiplague Yersin serum of various ages up to 10 and 11 years. He found the 11 year old serum useless and thought the loss of power might be due to the 0·5°/₀ phenol present. From his experiments he concluded that if serum be heated at 55° C. on each of three successive days for $1\frac{1}{4}$ hours and then be kept in the cold, in the dark and in the absence of air it will preserve its curative and protective properties unchanged for six years. Wladimiroff (1898) states that antiplague serum keeps up its strength for months and is not affected by passing through the tropics. Our tests do not go back far enough to enable us to speak of years, but we have the results of tests performed by Rowland and myself with an interval of some 18 months to 2 years between them.

The results are given in the following table:

Name of horse		Method of immunisation	Date of bleeding	Tested in 1910 by S.R. 1 c.cm. serum =	Tested in 1912 by A.T.M.C. 1 c.cm. serum =
Corbally	Y 65	Living cultures intravenously	22. 1. 9	10 M. L. D.	12 M. L. D.
Whiteface	Y 68	,, ,, ,,	15. 2. 9	5—10 ,,	6 not 12 M. L. D.
Kitasato	Y 72	,, ,, ,,	16. 12. 9	,, ,,	12 not 30 ,,
Whiteface	Y 74	L. I. V. + toxin	20. 12. 9	40 + ,,	60 not 90 ,,
Lightfoot	Y 75	Toxin only	29. 12. 9	84 ,,	90 M. L. D.
,,	Y 76	,, ,,	4. 1. 10	125 ,,	30 ,,
,,	Y 77	,, ,,	18. 10. 10	66 ,,	30 not 60 M. L. D.
Ramsay	Y 79	,, ,,	5. 11. 10	450 ,,	300 not 450 ,,

In these tests the M.L.D. of the toxin was about 0·7 mgm. or a little less, and the test dose was 2·1 mgm. The quantities of serum used were in the following descending scale: 1 c.cm., 0·5, 0·25, 0·1, 0·05, 0·02, 0·01 and 0·0066. This will explain why no values are given between 30 and 60 or between 300 and 450. These results show that plague antitoxin retains its value fairly well for 18 months to 2 years.

To ascertain the effect of heating on the antitoxin some small serum vials were filled with antiplague serum, sealed off in the flame and then heated in a water bath for $\frac{1}{2}$ hour at 56° C. on each of three successive days.

Name of horse	Date of bleeding	Antitoxin content in M.L.D.	
		Before heating	After heating
Wealdstone	3. 2. 12	75 M.L.D. per c.cm.	75 M.L.D. per c.cm.
Calcutta	3. 2. 12	30 ,, ,,	75* ,, ,,
Lemberg	3. 2. 12	30 ,, ,,	30 ,, ,,

* This appears to be an instance of a rat being hyperresistant. In the test before heating the rats which received $\frac{1}{25}$ c.cm., $\frac{1}{50}$ c.cm. and $\frac{1}{100}$ c.cm. of serum all died and in consequence it was not thought to be worth while to test the serum after heating beyond $\frac{1}{25}$ c.cm.

It is obvious that heating antiplague serum under the conditions described has no deteriorating effect upon the neutralising power for plague toxin.

Concentration of antitoxin.

As the titre of the serums obtained has not been very high an attempt was made to increase the value of a serum by the Gibson concentration process which has proved so successful in the case of diphtheria antitoxin. Five hundred cubic centimetres of serum were precipitated with an equal volume of half-saturated solution of ammonium sulphate. The precipitate was collected and extracted with brine. Eleven hundred (1100) c.cm. of brine filtrate were obtained and precipitated with 0·25% of acetic acid. The precipitate was collected, made alkaline with powdered washing soda and dialysed against tap water. When found comparatively free from salts the contents of the dialyser—200 c.cm.—were filtered first through chain cloth and then through a small Berkefeld filter candle.

The antitoxic values at the three stages were:

Original serum	500 c.cm.	90 M.L.D. per c.cm.
Brine extract	1100 ,,	45 ,, ,,
*Concentrated serum	200 ,,	150 ,, ,,

* Unfortunately the serum became contaminated in the dialyser and perhaps some of the loss may have been due to this cause.

There is thus an increase in the antitoxic value per c.cm. to the extent of $1\frac{2}{3}$, but on the whole a loss of 33% of antitoxin.

SUMMARY.

1. It is possible to obtain a plague antitoxic serum by means of injections of toxin obtained from the plague bacillus by Rowland's sulphate process. So far however the antitoxic value has not been high. The usual neutralising power has been 300—400 rat M.L.D. per c.cm. and on only one occasion was a value of 750 M.L.D. reached.

2. In an immunised horse one obtains a better response, as regards antitoxin production, if one uses an old weakened toxin instead of a fresh toxin.

3. Plague serum retains its antitoxic properties for months unimpaired.

4. Heating at 56° C. for ½ hour on each of three successive days has no effect on the antitoxin.

5. Plague antitoxin may be concentrated by the same process that is used for concentrating diphtheria antitoxin.

I desire to take this opportunity of thanking my colleague Dr S. R. Rowland for so generously placing at my disposal the large amounts of toxin which have been required. To my chief, Dr C. J. Martin, I am greatly indebted for much kindly and helpful criticism.

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ON THE TOXIN PRODUCTION OF THE DIPHTHERIA BACILLUS.

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(With 4 Charts.)

Everyone who has been occupied with the weekly production of diphtheria toxin in quantity has been struck with the great variations which may and usually do occur in the strength of the toxin from week to week. It is indeed extraordinary as Madsen remarks (1908, p. 83) that a bacillus should for a long time give good and fairly constant results when grown in one laboratory and yet fail to do so when grown in another laboratory in a medium prepared with the greatest care according to the formula used in the first laboratory. That it is not merely a question of medium is shown by the following experiment. A batch of bouillon was made up and inoculated in the usual way. Then half the flasks were placed in one of the two hot rooms we have here, and the other half in the other hot room. After nine days' incubation the cultures were treated as usual. Both lots were tested on the same day and it was found that while the M.L.D. of the one half was $\frac{1}{150}$ c.cm., that of the other was $\frac{1}{50}$ c.cm. Many and varied have been the procedures suggested for growing the diphtheria bacillus so as to obtain a highly potent filtrate and most of them have been tried in this laboratory but none can be depended upon to give a constant result. When discussing this point Dean (1907, pp. 467–468) gives the M.L.D. of a large number of toxins prepared under his direction, but they are not given in order as they were prepared and so one cannot get an idea as to how one batch differed from the next. I have not come across any such series in the literature of this subject and so it seemed that it would be of interest to put on record a two years' series of results in the order in which they were obtained—more especially as a study of this series gave more food for thought than was anticipated.

The bouillon used was prepared according to the method recommended by Dean (*loc. cit.* p. 466) and the details are given here for convenience sake.

Take 1 lb. of lean beef (silverside[1]) freed from fat, mince it and add 1 litre of tap water.

Boil 1–2 hours and filter while hot.

To filtrate add 2 % Witte's peptone and 0·5 % NaCl and steam 1 hour.

Neutralize with Na_2CO_3 solution using litmus paper as indicator and then add 7 c.c. $\frac{n}{1}$ NaOH solution.

Steam 1 hour and filter cold.

Distribute into flasks[2].

Sterilize in the autoclave (steam for 20 mins.—raise the temperature to 134° C. and then turn the gas off).

Each flask is inoculated with a small piece of the pellicle-growth from a flask which was seeded the previous week. The flasks are incubated at 36·5° C. On the third day a flask is taken out of the incubator and kept in the ice chest until required to inoculate the next batch at the end of the week.

On the ninth day the cultures are filtered through paper (S and S 597) and kept in Winchester quart glass-stoppered bottles, the surface of the toxin being covered with a layer of toluol. The bottles are placed in a cool dark cellar and the broth is shaken up each day for several days. At the end of a week, as all bacilli are dead, it is considered fit for testing, which usually takes place within a few days.

Chart No. 1 gives in the form of a curve the results obtained from week to week from September 1910 till July 1912. The ordinates represent the number of M.L.D.s per cubic centimetre and the abscissae the weeks.

As it was possible that toxin production might occur more quickly or more slowly during the summer I have tested the toxicity of cultures some of which had been grown for 3 and some for 14 days. In neither case was there any increase in strength over the 9-day growth.

Considered simply from the point of view of weekly variations there is nothing to which one need call attention as the curve only depicts what has been an universal experience and one for which no explanation is forthcoming.

But if the curve be studied as a whole it is manifest that there is a rise and fall in the size of the minimal lethal dose during the summer and winter respectively and this rise and fall is not gradual. At the end of summer there is a rapid increase in the production of toxin and then the strength of the toxin remains high until about the end of March when a drop occurs and the toxin production remains low until the autumn.

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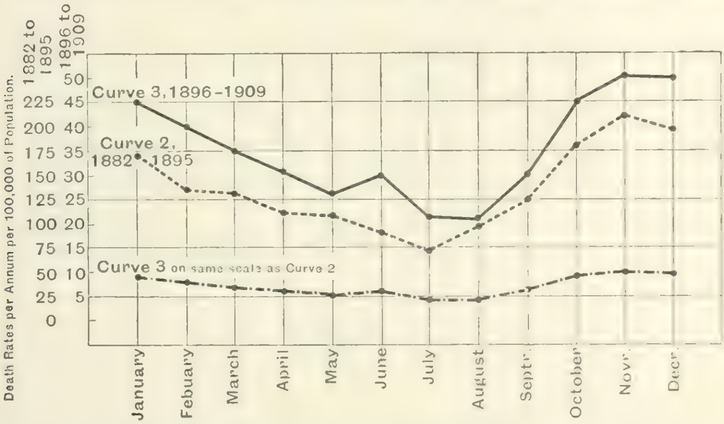
[1] The silverside of beef is almost invariably used but it is not the only part which gives good results. It does not seem to matter whether the meat is used quite fresh (*i.e.* while still warm) or two days or several days old.

[2] This broth is made up in batches of 10, 20, 30 or 40 litres and kept in bulk. When required it is distributed in quantities of about 300–350 c.cm. into 1 litre Erlenmeyer flasks, which are sterilized in the autoclave and inoculated as soon as the bouillon is cool enough.

It might be said that this seasonal variation was entirely due to changes in the resistance of the guinea-pig to the effects of diphtheria toxin—changes such as have been alluded to by Madsen (*loc. cit.* p. 90) and by Südmersen and Glenny (1909). While it is impossible to deny that this varying resistance may have some influence on the result still I do not think it can be accepted as the sole cause. From the data which we have at our disposal we cannot come to any definite conclusion and therefore we may leave this point for discussion at a future date.



Curves 2 and 3. Death rate from diphtheria and croup before (2) and after (3) the introduction of antitoxin.

What I more particularly desire to call attention to is the similarity between the curve of toxin-production and certain other curves showing the incidence of diphtheria in the human subject.

The first two curves, Nos. 2 and 3, are from a leaflet issued by one of the American Health Authorities. They show the death-rate from diphtheria and croup during two 14-year periods—one before and the other after the introduction of antitoxin. In the leaflet both curves are of course drawn to the same scale but I have altered the scale of the second curve in one particular simply so as to make the fact that there is a rise and fall more easily appreciated. We see that though a large drop in the death-rate followed the use of antitoxin still a rise and fall occurred at the same seasons.

Bearing in mind that these are the averages of 14 years and that in consequence abrupt changes must be to a considerable extent eliminated we cannot fail to be struck by the similarity between these two curves and the "toxin-curve"—especially in the rapid rise between July and November.

Curve 4 shows the number of cases of diphtheria notified week by week in the county of London and Curve 5 gives the number of cases of diphtheria admitted each week into the Metropolitan Asylums Board hospitals.

Now Südmersen and Glenny (p. 400) state that the toxin they studied apparently showed an increase in toxicity in one year compared with the previous year.

On directing our attention to this point we find that Curves 4 and 5 show that the number of cases of diphtheria notified or admitted into hospital was greater in the winter of 1911–12 than in that of 1910–11, and curve 1 shows that the strength of the various batches of diphtheria toxin reached and remained at a higher average level during 1911–12 than during 1910–11. We have then the curves of "toxin-production" and of "incidence in the human subject" agreeing in showing an annual as well as a seasonal variation. In fact the curves resemble each other in a remarkable and unexpected degree.

Naturally, we search for an explanation of this coincidence and we cannot find it as there are too many unknown factors in the problem. For instance, if we put these variations down to changes in the resistance of the animal organism, then we must admit that the susceptibility of all the people in the county of London has varied in much the same manner as that of our stock guinea-pigs. And on the other hand, if we consider them to be due to changes in the toxigenetic power of the bacillus, then we must allow that the offensive power of all the different strains of diphtheria bacilli in the county waxed and waned coincidently with that of our laboratory strain. Neither of these explanations is adequate and so we must leave the problem unsolved and be content with having drawn attention to the subject.

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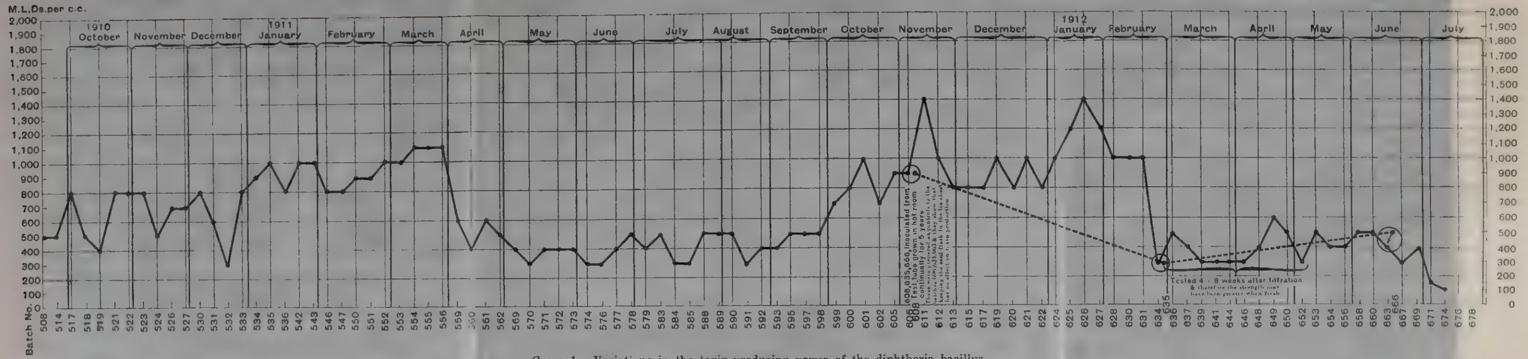
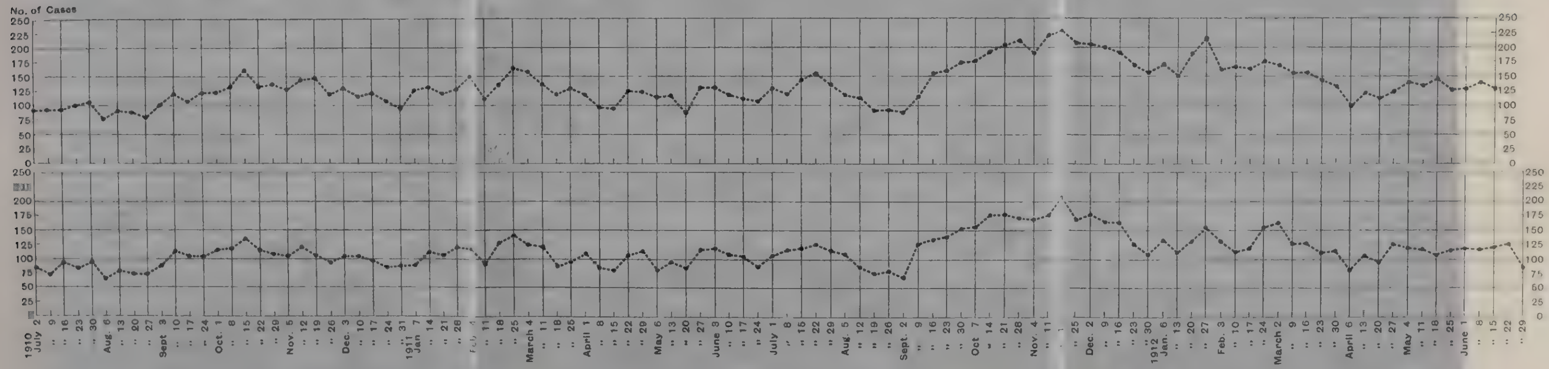


Curve 1. Variations in the toxin-producing power of the diphtheria bacillus.



Curve 4. Cases of diphtheria notified each week in the County of London.

Curve 5. Cases of diphtheria admitted each week into the Metropolitan Asylum Board Hospitals.

[From THE JOURNAL OF HYGIENE, Vol. XII, No. 4, January 10, 1913.]

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ON THE LOSS IN POTENCY OF DIPHTHERIA ANTITOXIN WHEN KEPT AT 36° C.

BY ALFRED MACCONKEY.
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(With 3 Charts.)

NUMEROUS papers have been published on the effect of heat on diphtheria antitoxin, but there appears to be no record of a series of observations carried out at a temperature nearly that of the human body. The object of this note is to fill in this slight gap in our knowledge.

A number of ordinary serum bottles were filled with serum and sealed off in the flame. Some were placed in the hot room at a temperature of 36° C., some were put in the ice chest and some in a cellar where the temperature varied between 6° C. and 16° C. From time to time a bottle was opened and the antitoxin-content estimated to within 10 units.

Three sera were treated in this way. In one case four bulk samples were also put up—100 c.cm. of serum in a 250 c.cm. flask plugged with a rubber cork. The serum in one of these flasks was covered with a layer of liquid paraffin. This flask and one of the other three were placed at 36° C., another was kept in the cellar, while the third was put in the ice chest.

The dates of the bleedings of the various horses and the results of the tests are given in the accompanying charts.

For convenience of comparison some values may be extracted from the charts.

Taking the sera kept in the ice chest and in the cellar, we find that the losses were approximately as follows:

	6 months		1 year	
	Ice chest	Cellar	Ice chest	Cellar
D 56 (mixture of 8 bleedings)	7 %	5 %	7 %	14 %
D 59 (mixture of 6 bleedings)	2 ,,	1 ,,	4 ,,	9 ,,
D 74 (single bleeding)	2 ,,	4 ,,		

These values agree quite well with those obtained by Anderson (1910) from an examination of 18 sera kept at 5° C. and 15° C. He found that the yearly loss of antitoxin was about 6 % at 5° C. and about 10 % at 15° C.

He also kept sera at "room-temperature" (this varied between 70° F. in winter and 95°–100° F. in summer) and found the yearly loss to be about 20 %.

Though his "room-temperature" was at some time above 36° C., it could not have been so for long judging by the results of keeping serum continuously at 36° C.

Loss in unitage of serum kept at 36° C.

	3 weeks	4 weeks	9 weeks	6 months	1 year
D 56	Nil	5 %	20 %	37 %	46 %
D 59	9 %	—	26 ,,	37 ,,	51 ,,
D 74 in bottle	14 ,,	—	22 ,,	35 ,,	—
D 74 in bulk	—	—	—	58 ,,	—
D 74 in bulk under paraffin	—	—	—	62 ,,	—

Anderson's (p. 25) maximum loss in three years at 15° C. was 36 % and at "room-temperature" 59 % whereas from my results there is at 36° C. a loss of about 36 % in six months and of about 49 % in one year. And there is an even more rapid decrease in value in serum kept *in bulk* at 36° C. as the loss in six months reached 60 %, which exceeds Anderson's *maximum* for three years at "room-temperature." A layer of paraffin over the surface of serum kept in bulk has no effect in diminishing the loss.

It is curious that two sera at first lost more in the ice chest than in the cellar.

In conclusion it may be said that when serum is kept in small bottles deterioration appears to take place on an average about six times as fast at 36° C. as in an ice chest.

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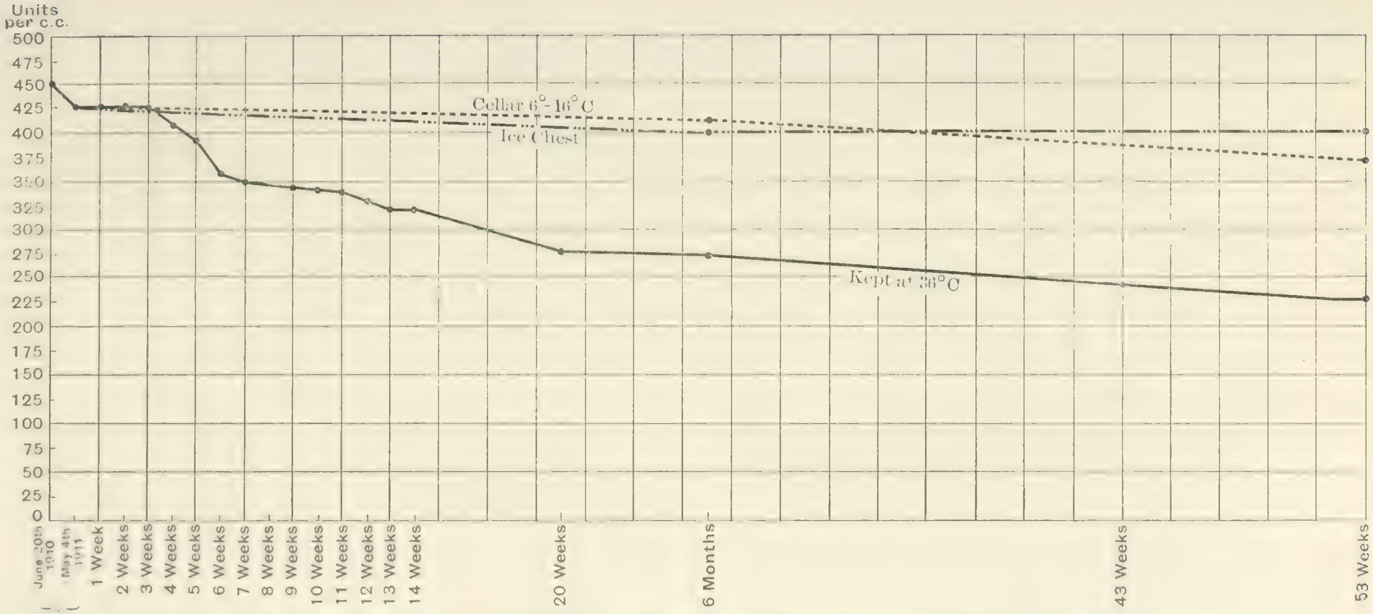

Chart 1. D 56. Bleedings—Common 13. I. 10; Huntress 17. I. 10; Cabby 31. I. 10; Brack 14. II. 10; Winchester 13. I. 10; Brougham 31. III. 10; Negress 13. I. 10; Negress 18. III. 10.

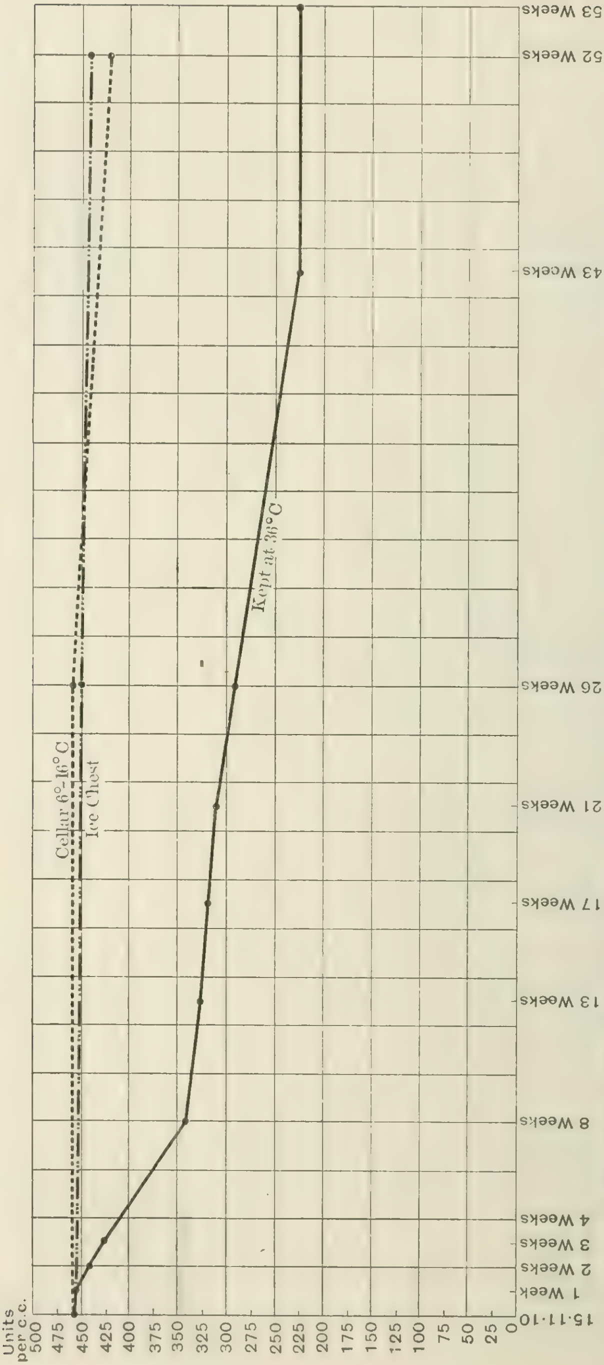

Chart 2. D 59. Bleedings—Negress 17. v. 10; Brougham 2. vi. 10; Truro 16. vi. 10; Oxford 17. ii. 10; Douglas 8. ix. 10; Oxford 16. vi. 10.

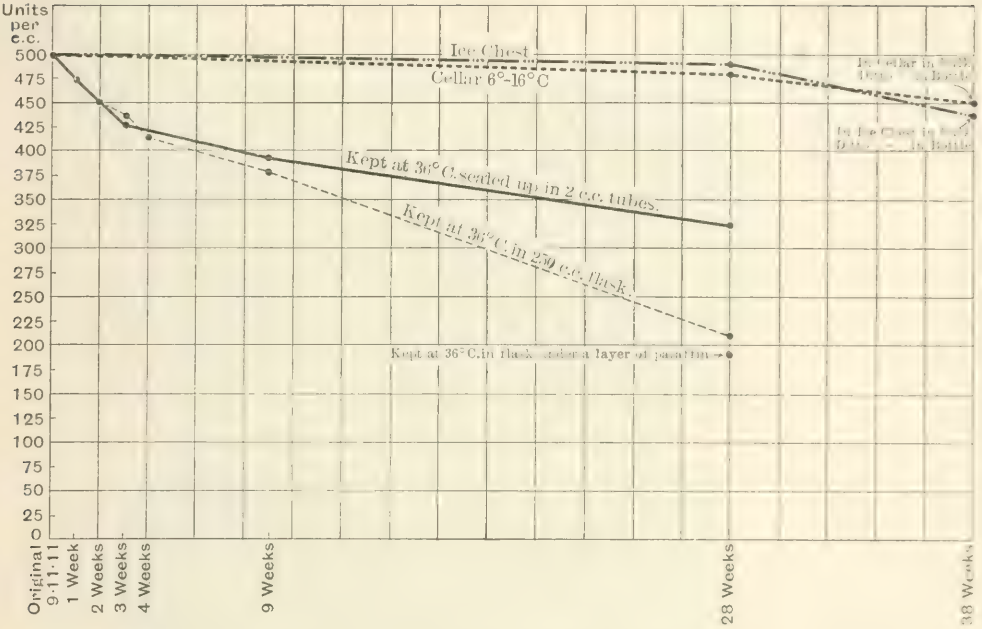

Chart 3. D 74. Oxhey, bleeding 22. II. 11.

Reprinted from the BRITISH MEDICAL JOURNAL, *January 4th and 11th, 1913.*

The Horace Dobell Lectures

ON

INSECT PORTERS OF BACTERIAL INFECTIONS.

DELIVERED BEFORE THE ROYAL COLLEGE OF PHYSICIANS.

BY C. J. MARTIN, M.B., D.Sc., F.R.S.,
Director of the Lister Institute of Preventive Medicine.

LECTURE I.

To intervene successfully in bacterial diseases of man and animals it is most important to know accurately the life-history of the parasite outside the host—how it leaves the old host, how it enters a new host, and where and how it passes the intervening period. If we are in possession of these facts, our efforts in the struggle can, at any rate, be intelligently directed and concentrated against the most vulnerable point of the enemy. The exercise of an adverse influence upon bacteria, once they have gained a footing in the body, has hitherto not been generally successful, although recent developments in chemotherapy have shown that there is ground for optimism in this direction.

In the early days of bacteriology, before the large number of pathogenic bacteria had been discovered and their peculiarities had been studied, it was generally assumed that bacteria once let loose from the body survived long periods upon inanimate objects and in water, soil, etc.; and efforts at prevention were directed to an indiscriminate disinfection of the belongings and surroundings of the patient. Some bacteria do, indeed, possess considerable powers of survival under such circumstances, and I would not be understood to deprecate measures of general disinfection. The majority of pathogenic organisms, however, are fortunately delicate creatures, and rapidly succumb to such adverse influences as drying and sunlight.

Another factor which must not be lost sight of is that many organisms which have acquired by selection the property of withstanding the adverse influence obtaining in the animal body fare ill in the competition with hardy saprophytic colleagues outside the body, and, if certain individuals do survive, these, whilst acquiring the capacity to live upon extracorporeal nutriment, diminish at the same time in pathogenicity. In other words, the conditions select a strain tending more and more towards saprophytism and away from parasitism. For these reasons it behoves us to be especially on the look-out for any machinery through the agency of which bacteria may be rapidly conveyed from sick to healthy, and their travels made easier for them.

In not a few diseases the infective agent usually passes almost directly from patient to patient, such as the organisms of syphilis, plague - pneumonia, diphtheria, measles, scarlet fever, small-pox, and numerous parasitic skin diseases. The spread of such highly infectious diseases can be dealt with by isolation and segregation of contacts, for their range is small.

Of recent years much attention has been bestowed upon the study of the rôle of insects in the transmission of disease. It is not my intention to deal with these discoveries as to the essential part played by insects in the transmission of protozoological parasites made by Smith and Kilbourne, Bruce, Ross; Reed, Carrol, and Agramonte, regarding the transmission of Texas fever, nagana, sleeping sickness, malaria of birds and animals, and yellow fever respectively, further than to point out that it was these researches which focussed attention upon the possibility of bacterial diseases being conveyed by a similar agency. In the latter case the insect plays a less essential part, and no particular phase in the life-history of the parasite takes place within it. I have accordingly, following a suggestion of my friend, Colonel Alcock, employed the term "porters" to describe this passive rôle of insects in the spread of contagion.

HOUSE-FLIES.

A deal of attention has been paid to flies lately in view of their possible influence in the dissemination of infection, more particularly in the case of such diseases as cholera, typhoid, and infantile diarrhoea, in which the infective agent escapes from the intestine and new infections are taken in by the mouth.

The first question is—Can the fly convey infection? Before referring to the numerous experiments which have afforded an answer, I will briefly refer to those points in the life-history, structure and habits of the house-fly which are of assistance in appreciating how it may play such a rôle. These subjects have been submitted to careful inquiry during the last few years, particularly in America and this country, and thanks to the observations of Lowne (1890), Howard (1912), Newstead (1907–9), Griffith (1908), Hewitt (1910), and Graham Smith (1910), we are now well acquainted with this insect, intimate knowledge of which was, until recently, curiously lacking.

The female fly lays about 120 eggs at each laying, and may produce four broods. Under ordinary conditions the eggs are mostly laid on horse, pig, or cow manure, but the excreta of almost any animal, or other fermenting refuse, suffices. The eggs are sausage-shaped with one end sharp, glistening white in colour, and about 1.5 mm. in length and 0.3 mm. in their greatest diameter. The eggs hatch in from three days to eight hours, according as the temperature ranges from 50° F. to 80° F. The larva is a little, active grub 2 mm. long, with a sharp anterior and blunt posterior end (Fig. 1). The larval stage lasts five days to three weeks, according to temperature. At the time of pupation the grub is 6 to 9 mm. in length. Pupation lasts five days to a month, according to temperature. Thus the whole cycle from laying of egg to emergence of the fly occupies ten days to two months, according as the weather be warm or cold. Shortly after emergence from the chrysalis the young fly spreads its wings, which soon harden, and flies away in search of food. The young female is ready to lay its first batch of eggs in about ten days, or even sooner in warm weather.[*] During winter a few flies survive in warm and secluded places. In the spring these start the next year's supply. The possible progeny of one female fly from April to September have been estimated at 10^{13}. Howard, of the United States Department of Agriculture, estimates that in forty days the descendants of one fly might number 12 million, or 800 lb. weight.

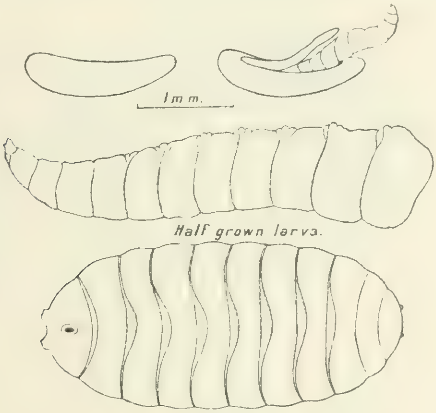

Fig. 1.

The only points in the structure of the fly it is essential to direct your attention to are the legs and feet and the general arrangement of the alimentary apparatus. These will be sufficiently obvious from the diagrams (Figs. 2 and 3). Please note that the feet are covered with minute hairs. The latter are, indeed, more numerous and finer than in the diagram, and extremely fine ones are also placed upon the pads (these are stated to secrete a sticky substance, by means of which the fly grips), the basal part of the claws, and the distal stiff hair which projects from the end of the last joint of the tarsus. Each leg is like a minute paint brush, which is applied to the surface of whatever it rests upon. When this is water the hairs do not appear to be wetted.

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[*] No doubt the cycle may be shorter or much longer under a wider range of temperature than that from which these records have been taken.

The essential parts of the alimentary canal are a gullet, stomach, crop, intestine and rectum. (See Fig. 3.) The gullet is prolonged to a minute opening between the flaps of the proboscis, halfway down which it is joined by the salivary duct. At the entrance to the stomach it is bifurcated, and one limb of the bifurcation is extended backwards to the bilobed crop. By a valvular apparatus at the entrance to the stomach, the insect can direct the liquid driven by the pump in its trunk into either the stomach or crop. The proboscis is a highly elastic muscular organ with universal movement. At the end are two flaps or labella (only one of which is shown), which it can open out like the leaves of a book, and apply the medial surfaces to the material it feeds upon. From the middle line or hinge minute chitinous channels pass outwards to the margin. These are seen in the diagram of the front view of the head of a fly (Fig. 4) in which the labella are shown opened out and facing forwards. These tubular structures are strengthened at frequent intervals by chitinous rings like a trachea, but are not complete tubes, being open to the surface by a minute linear channel with lateral bays in it. At the base of the trunk a number of muscle fibres are attached to the gullet by the peristaltic contraction of which fluid is pumped up from the mouth and propelled into the stomach or crop. The structural arrangement of the flaps of the trunk acts as a filter. Graham Smith (1911) is of opinion that solid objects

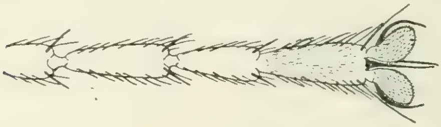

Fig. 2.

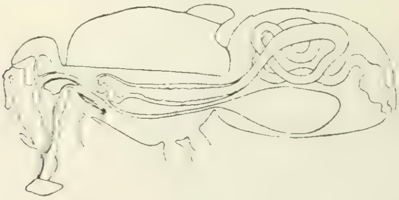

Fig. 3.

larger than 0.006 mm. seldom pass into the gullet. According to Nicoll (1911) the ova of such tapeworms as do not exceed 0.035 mm. in their smallest diameter may be swallowed. These must therefore get into the mouth direct. When feeding on a liquid, the fly applies the labella to the surface, and sucks the liquid through the "strainer" first of all into the crop. When this is full some goes into the stomach. In the case of solid material such as sugar, dried blood or sputum, the insect must first dissolve the material. This is done by pouring saliva upon it or more generally by regurgitating some of the contents of the crop (Graham Smith, 1910).

Graham Smith (1910), by feeding experiments with coloured syrup, found that the meal was first taken into the crop, and subsequently transferred to the stomach at leisure. The fly could, however, first fill its crop and then its stomach. In a quarter of an hour the meal had passed on to the upper third of the intestine, and in a warm incubator at 37° C. reached the rectum within an hour. The fly seems to keep some of the fluid in its crop for days.

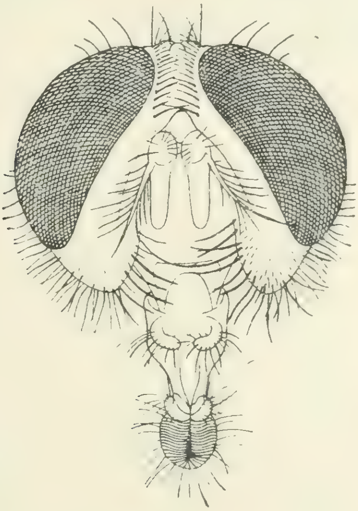

Fig. 4.

A well-fed fly deposits faeces abundantly. Graham Smith (1910) noticed flies to do this ten times in the first hour after feeding. A curious habit of flies, to which, so far as I know, attention was first drawn by Graham Smith, is the regurgitation of the contents of their crops. This has already been referred to as a means by which they are enabled to feed upon dry material soluble in water. They do this very frequently when walking over a clean glass, possibly with the idea of extracting nourishment from it. A fly, after a good meal, may often be seen blowing fluid bubbles from its trunk and sucking them in again, as in the diagram (Fig. 5), perhaps for practice.

From the above account it is clear that there are *a priori* reasons for suspecting the fly of carrying bacterial infection. Born in a dunghill, it spends its days flitting between the sugar basin, milk pan, and any faecal matter available. Its hairy, probably sticky, feet and the habit of regurgitating the contents of the crop and defaecating at frequent intervals suggests it to be an excellent inoculating agent for any bacteria it may pick up in the satisfaction of its unsavoury tastes.

That it does, indeed, operate in this way has been abundantly demonstrated. Flies which have wandered over cultures of organisms and afterwards been allowed to walk upon sterile agar plates leave a rich crop of germs as their footprints, which can be demonstrated by subsequent incubation. Castellani (1907) transferred yaws to monkeys in an analogous way.

The carriage of infection by the alimentary canal and its deposition by regurgitation or faeces has also been shown over and over again—Grassi (1883), Maddox (1885), Alessi (1888), Celli (1888), Sawtchenko (1892), Uffelmann (1892), Yersin (1894), Firth and Horrocks (1902), Manning (1902), Hayward (1904), Lord (1904), Chantemesse (1905), Buchanan (1907).

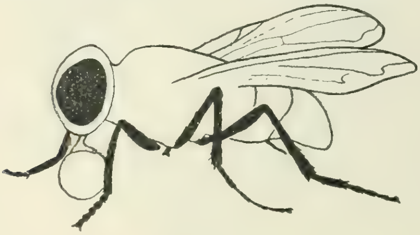

Fig. 5.

These modes are probably more important than the carriage of bacteria upon the exterior. Many pathogenic bacteria would soon die from desiccation on the appendages of the insect, and, at any rate, the number so conveyed is small compared to those contained in its crop and intestine.

Carriage within is certainly more lasting, for Graham Smith (1910), to whom we are indebted for the most thorough investigation of this subject, isolated typhoid and other bacilli from the intestinal contents of flies six days after feeding on material containing the organism under test. The faeces ceased to afford growths after two days when typhoid bacilli were the infecting organism used, but with a more robust organism, such as anthrax, which can protect itself from the effects of drying, the time was much longer.

The table below, taken from Dr. Graham Smith's report, summarizes the result of his experiments on the length of time after which various bacilli can be recovered from the outside and inside of flies fed on infected material. As the author is careful to point out, in these experiments gross infection was produced by feeding upon pure cultures, and they do not do more than indicate the duration of life of various pathogenic bacteria under favourable conditions.

Table showing the Longest Period after which Organisms were Recovered from Flies Fed on Cultures (*Graham Smith*).

Organism.	Legs.	Wings.	Head.	Crop.	Gut.	Faeces.
B. typhosus	—	—	—	—	6 days	2 days
B. enteritidis	7 days	—	7 days	8 days	7 days	-
B tuberculosis (culture)	—	—		3 days	16 days	13 days
B. tuberculosis (sputum)	—	—	—	—	7 days	5 days
Yeast	2½ hrs.	2½ hrs.	2½ hrs.	2 days	3 days	2 days
B. diphtheriae	5 hrs.	5 hrs.	5 days	7 days	5 days	2 days
B. anthracis (no spores)	2 days	—	4 days	5 days	3 days	2 days
B. cholerae	30 hrs.	5 hrs.	5 hrs.	2 days	2 days	30 hrs.
B. prodigiosus	8 days	12 hrs.	11 days	5 days	17 days	6 days
Anthrax spores... ...	20 days	20 days	0 days	13 days	20 days	13 days

Cao (1906), Bacot (1911), Ledingham (1911), and Graham Smith (1911) have further shown that in the case of larvae fed on material infected with various organisms, *B. pyocyaneus*, *B. typhosus*, and *B. anthracis* respectively, the infection may be carried through the chrysalis stage and recovered from the contents of the intestine of the fly after its emergence. Fortunately, however, according to Ledingham, *B. typhosus* leads a precarious existence in competition with the natural bacterial flora of the larvae and pupae.

There are numerous recorded instances in which the pathogenic organisms of cholera, typhoid, phthisis, anthrax, and plague have been recovered from the interior or dejections of flies which have been captured in the immediate neighbourhood of cases of the disease or, in the last two cases, of carcasses of animals dead of the disease. Cholera vibrios were isolated from wild flies under these circumstances by Tizzoni and Cattani (1886), Simmons (1892), Tsuzuki (1904); typhoid bacilli by Hamilton (1903), Ficker (1903), Faichnie (on seven occasions) (1909), Bertarelli (1910), and Cochrane (1912); tubercle bacilli by Spillmann and Haushalter (1886), Hofmann (1888), Lord (1904), Hayward (1904), Cobb (1905), and Buchanan (1907); anthrax by Cao, and plague bacilli by Yersin (1894) and Hunter (1906).

The spread of ophthalmia in hot countries has, on good grounds, been attributed to the agency of flies in carrying the Koch Weeks bacillus and the gonococcus from eye to eye—Budd (1862), Laveran (1880), Howe (1888), Axenfeld

(1907). The seasonal and local prevalence of ophthalmia corresponds with that of flies, and a visit to Egypt during the fly season is sufficient to convince one that this must happen.

Are Flies the Determining Factor in Epidemics?

Although, however, flies may be discovered with the infection of a number of diseases in or upon them, and by their habits may not unlikely serve as agents in transferring infection, it by no means follows that they are the determining factor of epidemicity in the case of cholera, typhoid, dysentery, etc. In the case of fulminating epidemics of typhoid and cholera associated with an infected water supply this is obviously not so.

Flies in Relation to Typhoid Fever.

The conclusion that fly transmission is the principal means of spread of typhoid in military encampments and stations has been arrived at by a number of competent observers, amongst them Veeder (1898), Reed, Vaughan, and Shakespeare (1900), in their report on the origin and spread of typhoid fever in the United States military camps during the Spanish war of 1898, and by Tooth and Calverley (1901), Smith (1903), Purdy (1909), and Wanhill (quoted by Purdy).

The sanitary arrangements of a military camp are not exactly those of the Ritz Hotel, and the prevalence of flies in late summer can hardly be appreciated by those who have not had the experience. The conditions are most favourable for transmission by flies. The circumstantial evidence against them is so strong as to have left no doubt in the mind of the American Commission that these insects play a large share in disseminating infection, for page 28 of their general statement and conclusions reads thus: "Flies undoubtedly served as carriers of infection."

An estimate of the fly population and its relation to admissions of enteric was made by Ainsworth (1909) in Poona. The observations showed that enteric had a very definite season, commencing at the end of the hot dry weather with the onset of the rains, and reaching a maximum a few weeks after their cessation. The abundance of flies increased also soon after the beginning of the rains, but earlier than the admissions for enteric, and, speaking generally, the fly curve antedated that for enteric cases by about one month.

Taking into account the incubation period for the disease, this fact is in agreement with the view of a causal relation between cases and flies in Poona. The number of observations is unfortunately small.

In considering the possible influence of flies in the spread of typhoid in a well sewered city, it must be remembered that the opportunities for flies to pick up the infection are vastly less than under the conditions of a military encampment, or even in rural surroundings. In large cities, no doubt, dejecta and urine from patients may be left available to flies, but the bulk goes promptly into the main drain.

The discovery, in cities like London and Manchester, of the majority of cases of typhoid, and their removal to hospital before the period when the excreta contain large numbers of bacilli, must also greatly reduce the liability to the dissemination of the disease by flies. The typhoid-carriers, however, remain.

Dr. Niven (1910) has made observations upon the prevalence of flies in Manchester since 1904. Twelve to thirty-four bell traps were served out to the inhabitants of small dwellings in different quarters of the town and the catch collected every few days by his inspector. Data have thus been obtained which are sufficient to afford information as to the general time relationship between fly prevalence and the incidence both of enteric and infantile diarrhoea.

It must be remembered, however, that the number of flies entrapped in twelve or seventeen rooms indicates only in a general way the prevalence of these insects in a large city, so that too much significance must not be attached to other than the main features of the record.

On pages 62 to 69 of Dr. Niven's paper are plotted the numbers of cases of enteric (commencement) and the diarrhoea deaths for each week of the year from 1891 to 1908. Whilst the latter show well-marked rises during some ten to twelve weeks of the summer, the seasonal prevalence of the latter is not striking. Speaking generally, the typhoid season in Manchester is considerably later than the diarrhoea season, and the enteric cases reach a maximum in late autumn, at a time when most of the flies have already disappeared.

The census of flies commenced in 1904, and, for the years 1904–9, coincides fairly well in time with the diarrhoea deaths if these are antedated ten days to allow for the average period ensuing between commencement of the disease and death.

If we assume that the same close time relation existed throughout, the observations taken as a whole seem to me to lend little support to the view that typhoid in Manchester is to any material extent dependent upon transmission by flies.

The time relation of fly prevalence and typhoid cases was also studied in Washington during 1908 by Rosenau, Lumsden and Kartle (1909) in collaboration with Howard, Entomologist to the United States Department of Agriculture.

In Washington in 1908 the flies (*Musca domestica*) appeared in considerable numbers in April, and reached a maximum in July. During August and September they steadily diminished to about one-third, and rose again in October. The relation of the two curves was not such as to suggest that flies play much of a rôle in the transmission of typhoid fever in Washington.

In a subsequent report on typhoid fever in Washington Lennoden and Anderson (1911), from an analysis of the incidence of typhoid upon the population using privies or yard closets, discovered that the greater prevalence of typhoid among these persons during the summer coincided with the fly season.

Fig. 6 shows the time relation of fly prevalence to enteric fever notifications for London during 1908. The data are taken from Dr. Hamer's (1909) observations giving the mean number of flies caught each day in 141 situations grouped around 9 centres. The observations being upon a larger scale, the sampling was presumably more representative of the fly population than the records in Manchester. I have antedated the records of the number of cases of enteric fever by three weeks—ten days for average lapse of time prior to notification, and eleven days for average incubation period. It will be seen that the number of cases of enteric fever rises steadily during the fly season, but that the rise continues independent of the fact that flies are rapidly diminishing, and reaches a maximum at a time when they have retired for the winter.

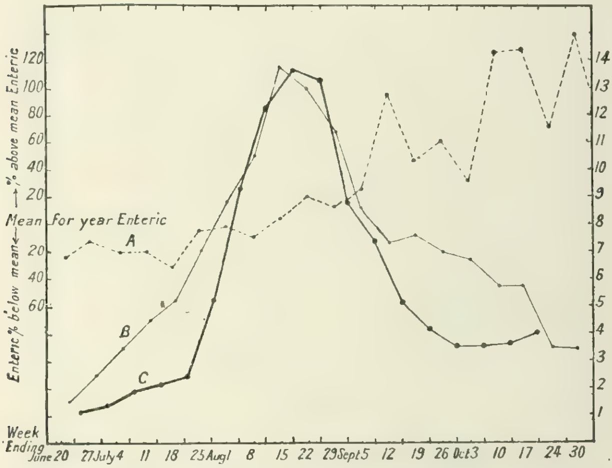

Fig. 6.—Relation in point of time between prevalence of flies, diarrhoea mortality, and cases of enteric fever, London, 1908. The deaths from diarrhoea have been antedated ten days; the cases of enteric notified have been antedated three weeks. A, Enteric cases. B, Flies caught per day ÷ 9,600. C, Diarrhoea deaths per week ÷ 20

As regards seasonal incidence of typhoid fever, 1908 is fairly typical of what has occurred in London during the last twenty years. As the weather became warmer, the number of infections with typhoid increased, and was not cut short by the fall in temperature usually occurring in September, but reached a maximum in October and November—that is, at a time when there are hardly any flies about. Whether, as Dr. Niven believes, increased fly prevalence is a factor in determining the increasing incidence in Manchester and London at the end of the summer, it seems to me impossible to say, but, taking into account the circumstantial evidence against the fly, this may not unlikely be the case. It is clear, however, that other causes are operative, and that epidemics of typhoid occur in these cities in the absence of flies.

This is in striking contrast to the observations in military camps both during the Spanish-American war and the Boer war. In both these instances, with the onset of cooler weather and the first frosty nights, the nuisance from flies was at once relieved, and the cases of enteric diminished shortly afterwards.

Flies in Relation to Infantile Diarrhoea.

As the infective agent of infantile diarrhoea has not been identified, no direct evidence that flies do, on occasion, convey the germs of this disease is available. It is probable that more than one etiological factor for infantile diarrhoea exists. Different organisms have been found to be particularly prevalent in the stools in different epidemics in various localities. In America some of the epidemics have been associated with the presence of a dysentery bacillus (Flexner's bacillus) in the stools, Wollstein (1903), Park (1903), Duval and Schorer (1903), Cordes (1903), Weaver and Tunnicliffe (1905). In other epidemics (in America) no dysentery bacilli were found, and an epidemic in Vienna investigated by Jehle and Charleton (1905) was not found to be associated with dysentery bacilli. Metchnikoff (1909) is of opinion that *B. vulgare* is the causative organism.

A search for evidence of a causal agent was conducted on a large scale by Morgan (1906, 1907, 1909) and his colleagues of the Lister Institute during the epidemic in London in the years 1905, 1906, 1907, and 1908. The search was restricted to aërobic bacilli which neither fermented lactose nor liquefied gelatin—that is, to a group which includes the dysentery, typhoid and paratyphoid, and Gaertner bacilli. Stools and autopsy material were examined from several hundred cases of the disease and from other children. A very large number of organisms were isolated and examined, but the only one whose prevalence was found to be related to infantile diarrhoea was a non-motile bacillus of this group, which was peculiar in that it failed to ferment mannite and is now generally known as Morgan's bacillus No. 1. This organism was not infrequently recovered from the organs in fatal cases and produced diarrhoea when fed to monkeys and young rats. It was rarely encountered in children not suffering from acute diarrhoea.

Morgan's bacillus has been found in flies captured from houses in which cases of diarrhoea occurred, but until its causal relation to the human disease is proven too much significance cannot be attributed to these observations.

We are therefore entirely dependent upon epidemiological facts in testing whether the belief in the dominant influence of the house-fly upon the occurrence of epidemics of infantile diarrhoea, a view which has been warmly exposed by many excellent observers, is valid.

THE RELATIONS IN TIME BETWEEN CASES OF DIARRHOEA AND PREVALENCE OF FLIES.

Unlike the case of typhoid, which has just been discussed, epidemics of infantile diarrhoea in towns exhibit a close time-relation with fly prevalence. Epidemic diarrhoea of children does not occur except during that season of the year when flies are abundant and active, and, as will be seen on studying the charts, the relation between fly population and diarrhoea cases is so striking as to suggest something more than a mere accidental dependence upon the same phenomena.

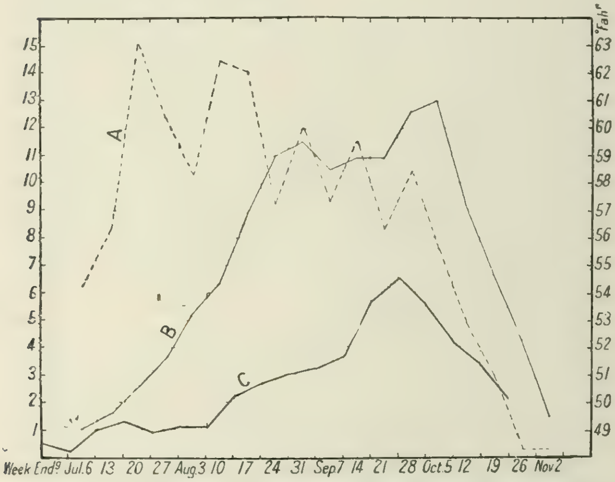

Fig. 7.—Dr. Hamer's observations on the relation in point of time between prevalence of flies and diarrhoea mortality in London, 1907 (163 fly-collecting centres). The deaths from diarrhoea have been antedated ten days. A, Weekly mean atmospheric temperature. B, Number of flies caught per day ÷ 10,000. C, Diarrhoea deaths per week ÷ 20.

The charts, Figs. 7, 8, 9, 10, and 11, show the relation of diarrhoea and fly-prevalence in time. The three first are derived from Dr. Hamer's observations in London in 1907, 1908, and 1909. Figs. 10 and 11 are constructed from Dr. Niven's (1910) observations in Manchester in 1904 and 1906. I have selected these particular years from the Manchester data because they represent what happened in a warm summer in which the number of cases was considerable.

The charts have undergone some manipulation at my hands. I have antedated the deaths by ten days, which, according to Dr. Niven's observations, represents the average period intervening between onset and death.

They ought no doubt to be antedated further to include the incubation period, but I have no data to determine what this should be. In the case of Dr. Hamer's observations, I have summed his tri-weekly observations and plotted the fly-catch for the whole week. In this way I have eliminated most of the irregularities and obtained smoother curves. I have also added the weekly mean temperature for London for the period comprised by Dr. Hamer's observations, and (in Fig. 8) for about two months previously.

The procedure adopted by Drs. Niven and Hamer to obtain data regarding fly prevalence was common in all cases. The number of flies caught on sticky paper, or entrapped in a series of domestic dwellings, generally in neighbourhoods inhabited by poor people, were recorded every two or three days. This estimate of the prevalence of flies, together with the deaths from diarrhoea, were then plotted as ordinates against weeks as abscissa. Dr. Niven's observations extend over five years, and refer to several districts in Manchester. Dr. Hamer's organization for the capture of flies was on a larger scale, and his observations were made at various parts of London during 1907 and 1908 and 1909, mostly in the neighbourhood of refuse and manure depôts, where flies are bred. Another excellent series of observations was made by Dr. Dudfield (1912) in Paddington during the summer of 1911. It concerns a smaller number of cases, but this enabled a more intensive study of many factors contributing to the epidemic to be made.

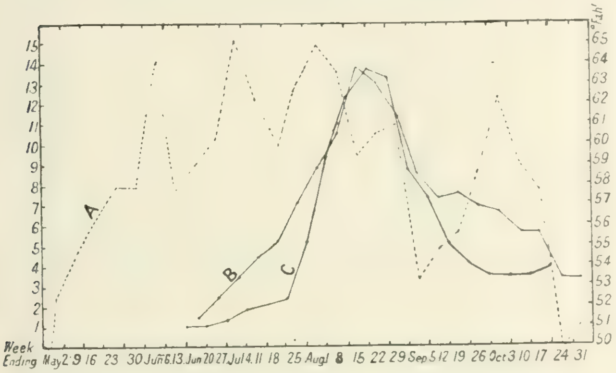

Fig. 8.—Dr. Hamer's observations on the relation in point of time between prevalence of flies and diarrhoea mortality in London, 1908. The deaths from diarrhoea have been antedated ten days. A, Mean atmospheric temperature for the week. B, Number of flies caught per week ÷ 9,600. C, Diarrhoea deaths per week ÷ 20.

The first point brought out by all these observations is the dependence of both the number of flies and the epidemic upon the cumulative effect of previous warm weather—as, for instance, is indicated by the earth temperature 4 ft. below the surface, a fact to which attention was first drawn by Ballard (1889). The curve for flies rises first, followed soon by that for cases. The latter is longer in getting going; this is particularly marked in the cool summer of 1907. Both flies and cases reach their

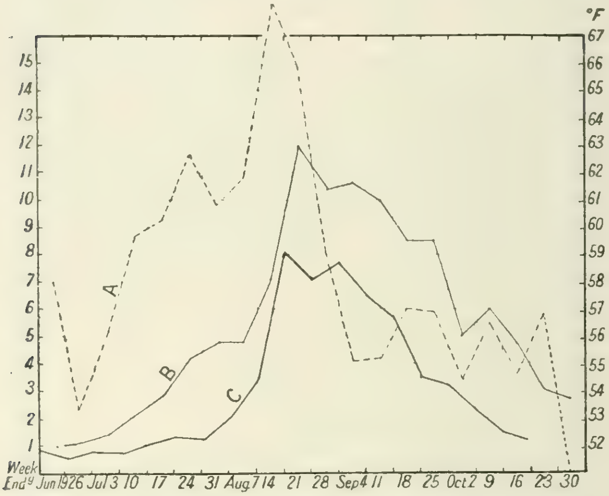

Fig. 9.—Dr. Hamer's observations on the relation in point of time between prevalence of flies and diarrhoea deaths, London, 1909 (thirty-five fly-collecting stations). The deaths have been antedated ten days. A, Weekly mean temperature of atmosphere. B, Number of flies caught daily ÷ 2,500 (weekly mean) × 4. C, Diarrhoea deaths weekly ÷ 20.

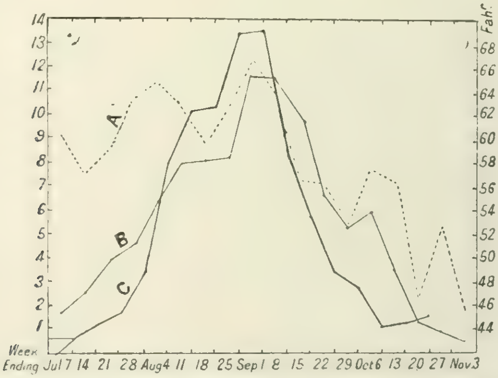

Fig. 10.—Dr. Niven's observations in Manchester, 1906. The deaths from diarrhoea are antedated ten days. A, Mean weekly temperature. B, Number of flies caught ÷ 2.000. C, Deaths from diarrhoea.

maxima about the same time. In 1907 the number of cases is diminishing whilst flies are still on the increase. In 1908 and 1909 both curves decline together, but that for flies more gradually.

A notable feature of the curve is that the same fly population is accompanied by a rise in the number of cases in early August and with a fall in early September. This fact has been quite properly emphasized by the critics of those advocating the importance of flies.

If we propose to regard the time relations of fly prevalence and diarrhoea cases as more than an interesting coincidence, we must find a satisfactory explanation for the above facts. Let us consider for a moment how the dependence of diarrhoea epidemics upon the accumulated effect of temperature might be explained.

The reason why the number of flies should be dependent upon this factor is obviously because the generation time (cycle from egg to egg) is dependent on temperature and requires three weeks or upwards in our climate. Months of warm weather are therefore required to produce any multitude of flies from the few surviving the winter.

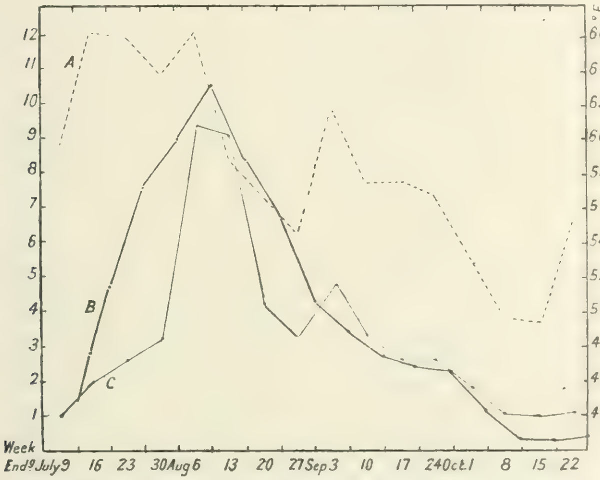

Fig. 11.—Dr. Niven's observations, Manchester, 1904. The deaths from diarrhoea have been antedated ten days. A, Mean weekly temperature. B, Deaths from diarrhoea ÷ 10. C, Number of flies caught ÷ 2,000.

Why the epidemic should exhibit this dependence is not obvious. Not knowing the etiology of diarrhoea, we must assume some sort of infective agent. From analogy with typhoid, cholera, acute food poisoning, and dysentery, we will suppose that we have to deal with some form of bacterium with which our aliments might become infected. In this case, would the dose of infection be dependent upon the accumulated effect of temperature during the previous three months? Assuredly not. The generation time of bacteria is so short—in the case of *Bacillus coli* it is one hour at 20° C. and half an hour at 30° C.—that the number present at any moment is to all intents and purposes dependent only upon the mean temperature during the last few days. To take a homely illustration, whether one's milk sours does not depend upon the temperature last month but last night.

Rate of Multiplication (Maximum Observed).

Days.	House-fly, August–September, 1907. Brighton.			*B. coli communis*, 20° C.
0	1 (Female).			1
	1st Gen.	2nd Gen.	3rd Gen.	
1	—	—	—	10^7 (10,000,000
2	—	—	—	10^{14}
5	—	—	—	10^{35}
10	—	—	—	10^{70}
20	100	—	—	
30	100	—	—	10^{720}
40	100	5,000	—	
50	100	5,000	—	
60	—	5,000	125,000	10^{1440}
	Total ...	...	150,000	

In the accompanying table I have set out the rate of multiplication of *B. coli* at 20° C. and that of flies inside a house during a summer in England. You will observe that one female fly might under these conditions possess 150,000 progeny in sixty days, but the bacteria derived from one organism become innumerable in two days. Unless, therefore, the effect of two months' summer weather be to change the nature of the organism so as to enhance its virulence, it is quite impossible to account for this striking characteristic of the epidemic by chance contamination of food supply by pathogenic bacteria. In a state of innocence regarding the etiology of the disease, we have no means of knowing whether any such change in virulence occurs under the influence of continued warm weather, but it is just conceivable.

On the other hand, we have excellent *a priori* reasons for supposing that flies could transmit the infective agent of diarrhoea. Any one familiar with the domestic *ménage* of the average working man on a hot summer day, with the baby sick with diarrhoea and other small children to care for, must realize that the opportunities afforded for fly transmission are adequate enough.

It seems to me, therefore, that before abandoning the view that diarrhoea is a bacterial infection, or making the gratuitous assumption that a long spell of warm weather is necessary to awaken pathogenicity, one must carefully examine the adequacy of the view that the dependence of the epidemic on accumulated temperature is to be accounted for by the operation of this factor upon the means of transmission.

We will now see whether this interpretation fits the rest of the facts.

The greater steepness of the rise of the diarrhoea curve is consistent with this view, for at first the opportunities for a fly to transmit the infection are small, but as the cases increase opportunities increase likewise.

As has been pointed out by Hamer and Peters (loc. cit.), if variation in the number of fly transmitters of an infective agent existing in the stools of patients were the sole factors concerned, the curve for diarrhoea cases should shoot up beyond and come down later and more slowly than that representing the number of flies, because the opportunities afforded to flies to pick up the infective agent are increasing *pari passu* with the development of the epidemic. This is, however, not what happens. On the contrary, the epidemic is arrested while flies are numerous and declines more quickly than the fly population. We are therefore confronted with the question, What arrests the rise in the epidemic wave?

Exhaustion of susceptible material has been suggested. This obviously plays a part in every epidemic, and the observations of Peters (1910) in Mansfield, where, under similar conditions, in one section of the town the epidemic was nearly finished whilst in another it was beginning, afford a demonstration that it is a factor of importance. As, however, small epidemics in cool summers exhibit the same feature, this cannot be entirely responsible for the arrest.

Another factor which must be taken into account is the atmospheric temperature at the time. A glance at the charts (Figs. 7, 8, 9, 10, 11), in which the weekly mean temperature is also shown, suggests that this is playing an important part, for the decline of the epidemic occurs immediately upon a considerable drop in mean temperature, and a diminution of fly prevalence often follows shortly afterwards. The influence of atmospheric temperature appears to be more marked upon the number of infections than upon the number of flies. Whereas in London, 1908 and 1909, following shortly upon the colder weather, both curves drop in a remarkably similar fashion; in the small epidemic of 1907 the number of flies still remains up and even increasing, whilst the epidemic is rapidly disappearing. The same general facts emerge from the Manchester observations. We might invoke the assistance of coincident exhaustion of susceptible material or diminished activity of flies, although still numerically sufficient, in explanation of this. They may indeed be operative, but I think that a completely satisfactory interpretation of the above facts, and also of the fact that, with a given number of flies, the epidemic wave is ascending during the early weeks of an epidemic and subsiding a month or six weeks later, is afforded by taking into account the influence of the variations in atmospheric temperature upon the rate of multiplication of the infective agent. Regard for a moment (Fig. 8) the chart expressing the results of Dr. Hamer's observations in London, 1908. Take the weeks ending August 25th and September 12th, in which approximately the same number of flies were caught. The mean temperature for the former week was 62.6° F.; for the latter it was 54.8° F., and it had been down to 53° F.

The atmospheric temperature is a factor to which insufficient attention has been given, since the origin of the epidemic showed but small relation to it. It is capable, however, of explaining why the epidemic wave rises in August and falls in September, although the number of flies may be the same. Assuming, for the sake of argument, some form of bacterial infection is the etiological factor in infantile diarrhoea, it would be in accordance with general experience to suppose that the chance of infection is determined by the quantity of the infecting agent imbibed.

This will depend upon the multiplication which has taken place in the infected medium prior to ingestion; this, in turn, is a function of the temperature at which the milk or other foodstuff has been kept. The temperature coefficient of bacterial multiplication is not higher than that governing ordinary chemical processes—namely, two to three times for 10° C.—but as bacterial multiplication proceeds logarithmically a comparatively small change in rate is sufficient to produce a large difference in the number of organisms contained in, say, an infected milk, if an interval of some hours elapses between inoculation and drinking. Thus if N *Bacilli coli* be inoculated in milk at 60° F. and 70° F. respectively, the number after twelve hours will be 126 N and 4,000 N respectively.*

We have, then, two factors which may contribute in varying degree to the decline of the epidemic.

1. A fall in temperature diminishing (*a*) the activity and number of supposed transmitters, and (*b*) the dose of infection which the child ingests owing to the effect upon the rate of multiplication of the infective agent.
2. The exhaustion of the more susceptible individuals.

Sometimes one, sometimes another of the above factors appears to predominate. For instance, we have the fact to which attention was drawn by Peters (1909), that in a much hotter climate, such as Australia, the epidemic often declines, notwithstanding the fact that the temperature may continue to rise for several weeks. In Fig. 12 below I have plotted from a paper by Stawell (1899) the monthly mean temperature and monthly diarrhoea mortality in Melbourne for the years 1895–96. In this city, where winter and summer succeed one another without the long intervening periods we have here, the epidemic lasts for six months instead of six weeks. It will be noticed that in 1895 the temperature continued to mount for two months after the summit of the epidemic. In Melbourne the decline cannot be due to diminished dose of the poison owing to slower rate of bacterial growth, but must be attributed to exhaustion of susceptible individuals or to the effect upon the presumed transmitting agents, the flies. In the absence of definite quantitative observations I cannot speak dogmatically, but, as far as my memory serves me, I think the number of flies diminishes in Melbourne, as in Washington, during the hotter weather. A monthly mean temperature of 70° in Melbourne indicates that there were a good many days with a maximum shade temperature of over 100° F., which in that locality is associated with extremely dry north winds. Such meteorological conditions are very fatal to flies both in larval and imago stage. On hot-wind days flies have to seek shelter out of the sun, or they rapidly become desiccated. For the same reason they succumb on an exposed window pane, and large numbers of dead flies accumulate on the window frame.

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* For the observations on which this calculation is based, see Dr. Janet Lane-Claypon's paper, "Multiplication of Bacteria and the Influence of Temperature thereon," *Journ. of Hygiene*, ix (1909), p. 239.

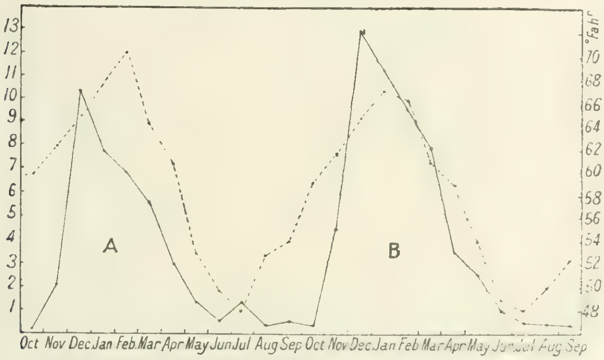

Fig. 12.—Diarrhoea mortality and mean monthly temperature, Melbourne, Australia. A, Summer, 1895–6. B, Summer, 1896–7.

The Relation of Fly Prevalence and Diarrhoea on Space.

No quantitative observations upon the relation between number of flies and the distribution of cases of infantile diarrhoea have, as far as I am aware, been made, but I may mention a few impressions by different observers for what they are worth. Nash (1909), who, whilst medical officer of health for Southend, made, during 1904, spot maps of the epidemic there, says: "The great majority of the deaths from diarrhoea were shown to have occurred in streets in proximity to brickfields, in which were deposited daily some thirty tons of fresh house refuse, which bred incalculable numbers of flies" directly the meteorological or seasonable conditions became favourable for their development.

Dr. Niven (1910, p. 45) is of opinion that the close correspondence between flies and cases of fatal diarrhoea receives a general support from the diarrhoea history of sanitary subdivisions of Manchester, and that the few facts available for the study of correspondence of flies and fatal cases in different subdivisions (Manchester) in the course of the same year also lends support. In his view no other explanation even approximately meets the case.

Peters (1910), in his very exhaustive study of the epidemic in Mansfield in 1908, paid especial attention to the possible part played by flies. From an analysis of his observations, he, I think justly, came to the conclusion that the evidence pointed to the fact that whatever part they might play as carriers from an infected household to a neighbour, flies did not bring infection with them from the manure heaps where they had been bred.

Peters constantly noted that the individual seemed to be a source of infection to the neighbours adjacent to the house within the precincts of which his excreta, if infectious, would most likely be deposited. This, he points out, could be most readily explained by the supposition that flies were the agents of dissemination of the infection, but from the frequent want of correspondence in different localities between numbers of flies and amount of diarrhoea, although not negativing it, gave no support to this view.

I doubt very much whether any evidence of great value could be obtained upon this point. Even supposing it to be true that fly-carriage is of first importance, I should expect that, if all the facts were known, a much higher correlation would be discovered between diarrhoea and carelessness with regard to disposal of excreta and protection of food from the visitation of flies, than between diarrhoea and fly prevalence.

Many of the facts which I have brought forward merely indicate some form of infective agent and do not necessitate recourse to the hypothesis that carriage by flies dominates the situation. I would point out, however, that—

1. The fly-carrier hypothesis is the only one which offers a satisfactory interpretation of the extraordinary dependence of the epidemic upon the accumulated effect of temperature.

2. That it offers a ready explanation of the spread of infection to neighbouring children who have no direct personal contact with the patient.

3. That the peculiarities of the relation in time between fly prevalence and the epidemic in different localities are not inconsistent with the view that fly-carriage is essential to epidemicity. No other interpretation which is at present forthcoming is nearly so satisfactory, and it is at least worthy to guide in the meantime our efforts at prevention.

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LECTURE II.

The Transmission of Plague by Fleas.

May I remind you that bubonic plague is not an infectious disease? The patient is a negligible source of danger to his surroundings, provided he does not develop a secondary pneumonia. The reason is that, even if the excreta do contain some plague bacilli, there is no mechanism available to convey them into a second human being, as pest is not easily contracted by feeding. From an epidemiological point of view bubonic plague must be regarded as a disease of rats, in which, under suitable conditions, the infection spreads from rats to man.

It would be impossible for me to put before you this afternoon the mass of evidence for the above statements. I have already surveyed it in opening the discussion on the "Spread of Plague" at the meeting of the British Medical Association at Birmingham in 1911 (Martin, 1911), and, moreover, it is now well known.

It was difficult to explain how the bacillus was transferred to man from the rat, especially as man-to-man infection had been shown to be negligible. On epidemiological grounds, Ogata (1897), Simond (1898), and Ashburton Thompson (1900) came to the conclusion that the agent must be some form of insect, and for various reasons choice fell upon the flea.

You will naturally inquire why, if the flea is to be considered an agent of transmission from rat to man, does it not transmit from man to man? The answer is quite satisfactory, but I will, with your permission, postpone it until we have considered the case for carriage from rat to man.

If the blood of the animal contain a sufficiency of plague bacilli, some will obviously be taken in by a flea whilst feeding, and Ogata (1897) found that crushed fleas taken from a plague-infected rat produced the disease when injected into mice. This experiment was repeated with success by Simond (1898) and Tidswell (1900).

The Mechanism by Means of which the Flea might Infect a Healthy Animal.[*]

The blood is sucked up from the wound made by the pricker. This structure is composed of three parts—the epipharynx and the two mandibles (Fig. 14). The apposition of the three forms a fine tube (Fig. 15), up which the blood is drawn, and passed down the gullet into the stomach by successive waves of contraction from before backwards of the muscles actuating the chitinous pharynx. The stomach is a pear-shaped organ occupying a considerable part of the abdomen of the insect. The internal economy of a flea and the arrangements of the mouth parts may be gleaned from the diagrams (Figs. 13, 14, and 15), which are borrowed from the Reports of the Commission.

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[*] In dealing with the agency of fleas in the spread of plague I shall draw largely upon the work accomplished during the last few years by the Commission for the Investigation of Plague in India, with which I have had the honour to be associated. The Reports of the Commission have been published as special numbers of the *Journal of Hygiene*, 1906 to 1912.

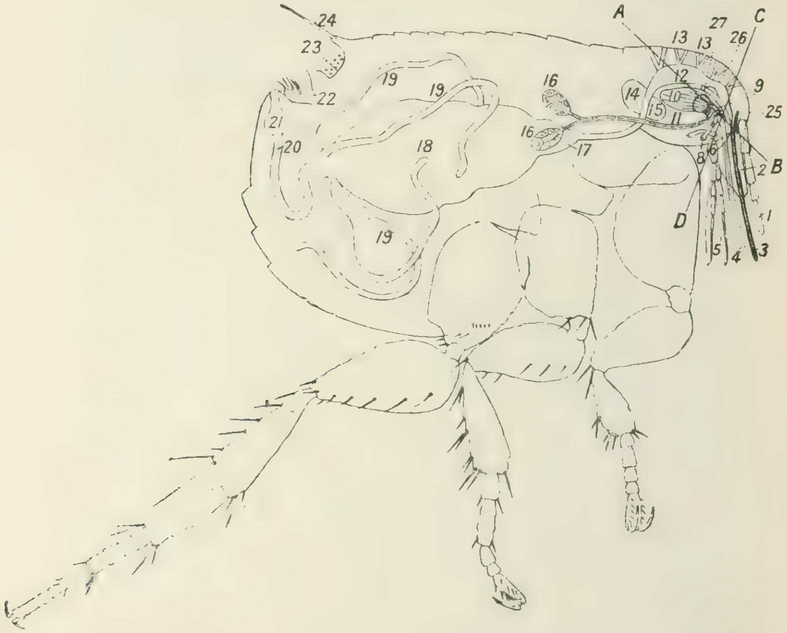

Fig. 13.—Diagrammatic mesial sectional view of *Xenopsylla cheopis*. 1, Maxillary palp; 2, maxilla; 3, epipharynx; 4, mandibles; 5, labium; 6, undivided portion of labium; 7, basal portion of labium; 8, basal portion of mandibles; 9, salivary pump; 10, hypopharynx; 11, salivary duct; 12, aspiratory pharynx; 13, muscles operating the aspiratory pharynx; 14, supraoesophageal ganglion; 15, suboesophageal ganglion; 16, salivary glands; 17, gizzard; 18, stomach; 19, Malpighian tubules; 20, rectum; 21, rectal glands; 22, claspers; 23, pygidium; 24, antepygidial bristle; 25, termination of dorsal contour of epipharynx; 26, mouth; 27, ligament.

The average capacity of a rat-flea's stomach was found by the Commission for the Investigation of Plague in India (Report, 1907, p. 397) to be 0.5 c.mm., and the number of bacilli in the blood of a plague-infected rat before death anything up to 100,000,000 bacilli per cubic centimetre. If, therefore, a rat-flea imbibed the blood of such a rat, it would receive into its stomach 5,000 germs.

Evidence of Multiplication of Bacilli in the Stomach of the Flea.

The Commission fed fleas on plague-infected rats until the death of the latter and afterwards on healthy animals, a fresh animal being supplied each day. Each day a number of the fleas was dissected and the stomach contents examined as to the presence or absence of plague bacilli. In 5 to 30 per cent., according to the time of year, plague germs were found up to the sixth day, and in one instance on the twentieth day (Reports, 1907, pp. 398–405). The bacilli were often present in immense numbers, far more numerous than ever seen in blood, and massed together as in culture. We have good evidence in this observation that multiplication of plague bacilli may take place in the flea's stomach. During the season of the year when the epidemic occurs the proportion of infected fleas for the first four days after removal from the plague rat was 43 per cent., on the sixth day there were 15 per cent., on the eighth day 16 per cent., and on the twelfth day 9 per cent. In the non-epidemic season only 5.2 per cent. were infected during the first six days.

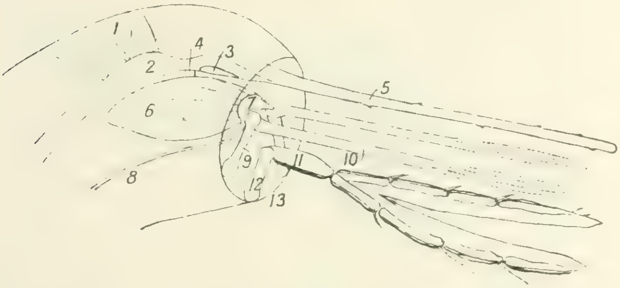

Fig. 14.—Diagram of the mouth parts of the flea. 1, Muscles operating aspiratory pharynx; 2, aspiratory pharynx; 3, mouth; 4, ligament; 5, epipharynx; 6, hypopharynx and muscles operating salivary pump; 7, salivary pump; 8, salivary duct; 9, basal element of mandible; 10, mandible; 11, labium; 12, basal element of labium; 13, perioral ring.

The Distribution of Plague Bacilli in the Body of the Flea.

The blood on completion of the digestive process in the stomach passes into the rectum of the flea as a thick, slimy, dark-red mass, and appears at the anus as minute, dark-red or black, tarry droplets. It was demonstrated by cultural and microscopical examination that the rectal contents were often crowded with plague bacilli.

Fleas, taken from plague rats at intervals varying from a few hours to several days, were dissected, and the various parts of the body examined for the presence of bacilli. In not a single instance were plague bacilli observed outside the organs already mentioned. No infection of the body cavity was seen, and although particular attention was paid to the salivary glands, nothing at all resembling a plague bacillus was ever detected in them.

How the Flea may Transmit its Infection to the Healthy Animal.

Several methods of transmission are possible, such as (*a*) the animal eating the infected fleas; (*b*) the mechanical conveyance of the bacilli by the pricker; (*c*) the regurgitation of the stomach contents down the pricker; (*d*) the deposition of the faeces on the skin, the bacilli being subsequently rubbed in by scratching.

This last method is the only one which has been proved capable of bringing about infection. A well-fed flea deposits a considerable amount of faeces in a surprisingly short time, and it was proved by both the Commission for the Investigation of Plague in India (Report, 1907, p. 418) and Verjbitski (1904) that wounds made by the pricker might afford a sufficient avenue for the entrance of bacilli when liquid containing them was gently applied to recent bites.

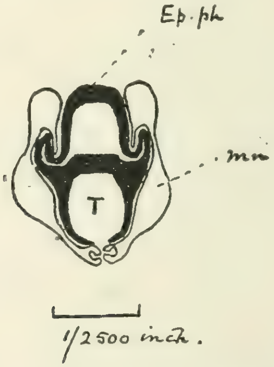

Fig. 15.—Transverse section of piercing organ of flea Ep.ph., Epipharynx. Mn., Mandibles. T, Tube through which blood is sucked.

Whilst, however, experiments have shown that infection may be brought about in this way, the possibility of infection by a regurgitation from the stomach preliminary to sucking blood cannot be excluded.

Proof that Plague is Carried from Animal to Animal by Fleas.

Simond (1898) infected one rat from another by placing them in a bottle together with twenty fleas. The second, uninfected rat, was enclosed in an iron box with a grating, so that the two animals could not come into contact. Gauthier and Raybaud (1902, 1903) repeated Simond's experiments under better controlled experimental conditions. They employed a cage divided in the middle by two wire grills 2 cm. apart, placed in a glass jar. In one compartment was placed an inoculated white rat on which had been deposited a dozen fleas captured upon rats from ships in the harbour at Marseilles. When the inoculated animal died a healthy rat was placed in the second compartment, and after some hours had elapsed, during which the fleas transferred themselves from the dead to the living animal, the cadaver was removed. Gauthier and Raybaud succeeded five times in conveying the infection from one rat to another; the number of negative experiments is not stated. An examination of the stomachs of the fleas found upon the septicaemic animals showed the presence of *B. pestis.* The fleas used in their early experiments were not identified. Tidswell (1903) made further attempts to convey plague from rat to rat by the agency of fleas, but was unable to do this.

Verjbitski (1904) carried out an extensive series of experiments on the flea-transmission of plague in St. Petersburg. He showed that the plague bacilli could be recovered from the stomach contents of fleas six days after they had fed on plague rats, and that the faeces of such fleas contained the *B. pestis* for about the same time. He succeeded in infecting rats with fleas from diseased rats fifteen times in seventy-six experiments. In each experiment ten possibly infected fleas were allowed to feed on the rat. Verjbitski also made experiments similar to those of Gauthier and Raybaud described above, and out of forty experiments, using ten fleas apiece, infection was conveyed in four.

Liston (1905) pointed out that the common flea infesting rats in India was not *Ceratophyllus fasciatus* or *Typhlopsylla musculi*, as in Europe, but a non-pectinated flea identified by Rothschild as *Xenopsylla cheopis.* Liston observed multiplication of the plague bacillus in the stomach of this flea. Although his experiments on transmission, which were made by allowing fleas to bite first an animal suffering from plague and subsequently a healthy animal, were not successful, he brought forward much interesting and valuable circumstantial evidence in favour of the view that plague is epidemiologically thus spread. He showed that X. *cheopis* takes readily to another host, for example, guinea-pigs and man, when rats are not available.

The possibility of the rat-flea carrying plague from one rat to another was demonstrated in a considerable series of experiments by the Commission for Investigation of Plague in India (Report, 1906, pp. 435–450). A glass case was used containing two wire cages side by side, each standing in a tin tray which collected the urine. Both trays were filled with sand in order to provide dryness and shelter for the fleas. Each cage was furnished with a lid through which the rats were introduced and food and water given to them, and the whole apparatus was covered in with fine muslin to prevent the escape of the fleas. A rat placed in one of these wire cages could not come in contact with a rat in the other cage, nor with its urine or faeces.

The method of experiment was as follows: A plague-infected rat and a number of rat-fleas were placed in one of the wire cages. After the rat died, a fresh healthy rat was put into the other cage, the corpse of the infected rat being left in for twenty-four hours longer.

Sixty-six experiments with English white rats and with Bombay wild rats were done in this way, with the result that 30 healthy rats contracted plague; fleas formed the only apparent means of transmission of the bacilli from rat to rat.

In order to exclude aërial infection a second series of experiments was carried out, in which fleas were taken from a rat which had died of plague and placed on a fresh rat in a clean flea-proof cage of similar construction to that already described, but containing only one wire cage. Out of 38 experiments 21 successful transmissions were obtained in this way.

The Importance of Flea-Transmission in Epizootics.

The following experiments, which had as their object the determination of the relative importance of the Indian rat-flea, *X. cheopis*, and of actual close contact in the absence of fleas in the dissemination of plague from animal to animal, were carried out by the Commission (Reports, 1906, pp. 450–467; 1907, pp. 421–436; 1910, pp. 315–335) in a series of small cabins, which were built especially for this purpose. In these animal houses plague-infected animals, rats or guinea-pigs, were kept in close contact with healthy animals. They ran about together in a confined space and ate out of the same dishes.

The plague-infected animals were allowed to die, and the corpses were not removed until some time after death. In some instances the concentration of infection was very great, in one case 21 infected animals being at one time in contact with 25 healthy ones. The cabins were never cleaned out, so that the animals ate food contaminated with the faeces and urine of their infected companions. In the experiments with rats, the healthy frequently ate the carcasses of the infected ones introduced.

Sixty-six series of experiments were carried out, each involving about 40 to 70 animals. In 35 experiments fleas were present, and 31 were control experiments in which no fleas were introduced. In all the control experiments not one of the healthy animals contracted plague, whereas in those cases where fleas were present an epizootic occurred, varying in extent and rate of spread according to the number of fleas present.

Some interesting experiments (Reports, 1906, p. 461) were also carried out in the animal houses that had been used for some of the previous experiments. Guinea-pigs and monkeys were introduced into them for one night. Some of the animals were in cages placed on the floor, and others in cages covered by fine muslin, gauze, or surrounded by a layer of sticky fly-paper, 6 in. wide—that is, the simplest precautions were taken to exclude fleas.

Of 13 unprotected monkeys, 6 died: of protected, none. Of 24 unprotected guinea-pigs, 18 died; of protected, none. The animal houses remained infective three weeks after the last animal had died of plague. This period corresponds with the maximum time fleas fed on a plague rat have been shown to remain infective when placed on a series of healthy animals.

Experiments in Plague Houses.

The Commission subsequently (Reports, 1906, pp. 467–482, and 1907, pp. 436–446) repeated similar experiments in plague houses in Bombay. The infectivity of 142 houses where cases had occurred or rats had died was tested by placing a guinea-pig there overnight. The guinea-pig was subsequently segregated, and in 31 cases it died of plague.

Ninety-two experiments were made in which two similar animals—monkeys, guinea-pigs, or rats, the one protected by gauze or sticky paper, the other not protected—were placed in suspected houses; 15 unprotected animals died; none of the protected.

On ninety-six occasions fleas taken off guinea-pigs which had spent the night in a suspected house were transferred to a healthy animal. In 26 cases the second animal died of plague, although not infrequently the guinea-pig from which they had been removed escaped infection. The conclusion that in a plague-infected house the infection is due to infected rat-fleas, and not to an infection of the soil or the air, seems to me abundantly justified.

Fleas as the Agents of Transmission from Rat to Man.

In considering how far the results just detailed can be applied to man, we enter at once upon less secure territory, because it is impossible to put conclusions to the test of experiment. The only measure of the correctness of such an interpretation is its adequacy to interpret all the known epidemiological facts concerning the spread of plague.

The justice of applying the results of these animal experiments has been severely criticized in several quarters, and particularly by Galli-Valerio (1907), on the ground that rat-fleas do not bite man. It is true that, speaking generally, different animals harbour specific parasites; but the more we learn upon this subject the clearer it becomes that such specificity is not so sharply defined as was imagined, and that a particular flea, although commonly confined to a few hosts of allied species, may frequently be found in considerable numbers upon quite different animals. Moreover, it has become abundantly clear that a flea, although exhibiting a decided preference for one species, will, if hungry, betake himself to animals of widely different type in the absence of its proper host.

Table showing Results of Feeding Experiments with Ceratophyllus fasciatus.

	Subject.								Summary.	Rat.	Rabbit.
	C. J. M.	H. C.	A. M.	J. H. S.	H. Y.	G. F. P.	H. W. A.	S. R.			
Total number of experiments ...	161	118	39	33	52	51	40	23	517	101	32
Positive	106	65	30	12	36	25	19	15	308	59	23
Negative	55	53	9	21	16	26	21	8	209	42	9
Per cent. positive	65.8	55.1	76.9	36.4	69.2	49.0	47.5	65.2	59.6	58.4	71.9

The common flea found on rats in India and other warm parts of the world* is *X. cheopis.* This flea is a non-combed species, and not unlike the human flea, *Pulex irritans*, in appearance. That *X. cheopis* readily feeds on man was observed by Tidswell (1903), Gauthier and Raybaud (1903), and Liston (1905). The Commission for the Investigation of Plague in India kept *X. cheopis* alive for four weeks upon an exclusively human diet, and my colleague Mr. Bacot has bred them for years on the same regimen.

With regard to the readiness with which *Ceratophyllus fasciatus*, the common rat-flea in Europe, attacks man, considerable divergence of opinion has hitherto existed. According to Wagner (quoted by Tiraboschi, 1904, p. 180), Tiraboschi (1904, p. 266), and Galli-Valerio (1907), this flea does not bite man. On the other hand, Gauthier and Raybaud (1903 and 1909) and McCoy and Mitzmain (1909) found that when hungry it fed on man with readiness.

Dr. Harriette Chick and I (1911) have made some hundreds of experiments on this question, and are at a loss to understand the negative conclusions arrived at by Tiraboschi and Galli-Valerio. Under the conditions of our experiments *Ceratophyllus fasciatus* fed upon man as readily as upon a rat. Nevertheless, it is doubtful whether *Ceratophyllus fasciatus* is attracted to man as readily as is is *X. cheopis*. Compared with the latter, it is a poor jumper.

We also made 107 experiments with *Ctenopthalmus agyrtes*, a flea common on *Mus decumanus* in this country, when living in fields, ricks, or barns, and 122 experiments with the mouse-flea, *Ctenopsylla musculi*. Under the conditions of the experiment the former would not bite man at all, and the latter only very occasionally. This latter experiment is interesting, because mice, although dying of plague, have not been found to be associated in the same way as rats with the origin of plague epidemics.

The Capability of Flea Transmission to Interpret the Epidemiological Facts regarding Bubonic Plague.

(1) Poverty, dirt, storage of grain in the bedroom, accumulation of refuse, and insanitary conditions generally, have been shown to be effective in so far as they lead to the support of a large rat population in close association with human beings. All these conditions also increase the population of rat-fleas, and man's accessibility to them.

(2) In 75 per cent. of cases the situation of the bubo is such that the bacillus must have obtained entry at some superficial area of the body. This is also consistent with infection by fleas

(3) A number of instances have occurred during the last ten years in India in which the introduction into a distant village of the effects of a person dead of plague has been followed after an interval of about a week by mortality amongst the rats, and subsequently by an

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* The distribution of rat-fleas has recently been dealt with by Dr. Chick and myself in the *Journal of Hygiene*, vol. ii, p. 129 (1911).

epidemic of plague. The same sequence has also taken place after the visit of an individual who had worked or resided in a house where some of the inmates had suffered from plague, although the stranger himself did not suffer from the disease.

These observations are better explained on the assumption that the infection was transported in the bodies of fleas contained in the clothing or upon the person of the stranger than by any other suggestion that has been put forward, for at the earliest opportunity rat-fleas would betake themselves to the rats, and could thereby start an epizootic. That such transmission of rat-fleas does actually happen has been proved by the Commission for the Investigation of Plague in India, members of which frequently carried away rat-fleas upon their persons and clothing when visiting native quarters in the course of their scientific duties.

(4) Perhaps the most striking feature of epidemics of bubonic plague is the marked seasonal prevalence of the disease amongst rats and amongst men. In places where it is endemic the epizootic, followed by the epidemic, starts at or about the same time each year, grows, declines, and more or less disappears. As one passes away from the Equator the plague season becomes later. In Bombay the height of the epidemic is in March, in Lahore in April, in Jhelum in May, in Rawal Pindi in June, and further north in July, August, and September. The epidemics of London in 1665, and of Marseilles in 1720, reached their maximum in September. The effect of latitude in determining the season when plague flourishes is analogous to that upon the flowering and seeding of a plant, and indicates dependence upon some biological factor. Within the tropics, where temperature variation is less and the seasons are determined by the prevailing winds and rainfall, the epidemic season is also well defined, but varies in different localities.

The seasonal incidence of plague is not due to any periodicity in the breeding of rats. In India, in localities where the latter was investigated, it bore no relation to the season when plague occurred. It was found by the Commission (Reports 1908, p. 266; and 1910, pp. 446 and 483), however, that the epidemic season coincided with the period of greatest flea prevalence. In Bombay, Belgaum, Poona, and two Punjab villages the Commission undertook a census of rat-fleas upon captured rats, in the course of which the fleas on 150,000 rats were recorded. In each case the observations extended over more than one year.

The analysis of the figures showed a seasonal variation in the number of rat-fleas in all the localities. The average number per rat varied in Bombay between 3 and 7, in Poona between 2 and 11, in the Punjab between 2 and 12, and in Belgaum between 1 and 17. The interesting point is that in all the places examined plague is epidemic when the average number of fleas is well above the mean, and the height of the epidemic corresponds fairly closely with the season of maximal flea prevalence. This coincidence is brought out in the attached charts (Figs. 16 and 17) borrowed from the reports, which show the variations in the number of fleas per rat and number of deaths from plague each week for Bombay and Belgaum respectively.

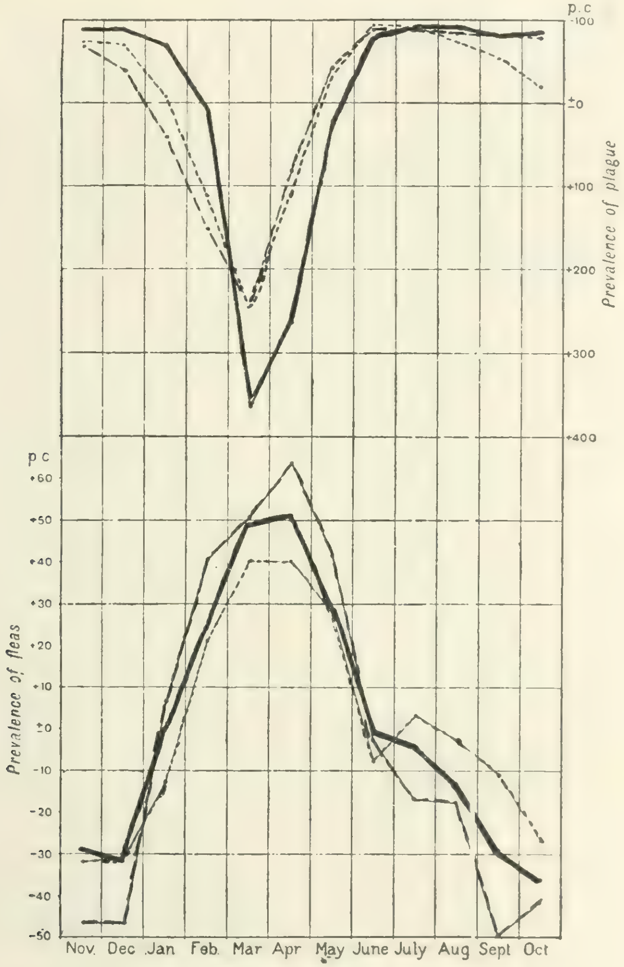


Fig. 16.—Prevalence of fleas in Bombay.

━━━ Human plague.
– – – Plague in *M. decumanus*.
------ Plague in *M. rattus*.
━━━ Fleas on all rats,
– – – Fleas on *M. decumanus*
------ Fleas on *M. rattus*.

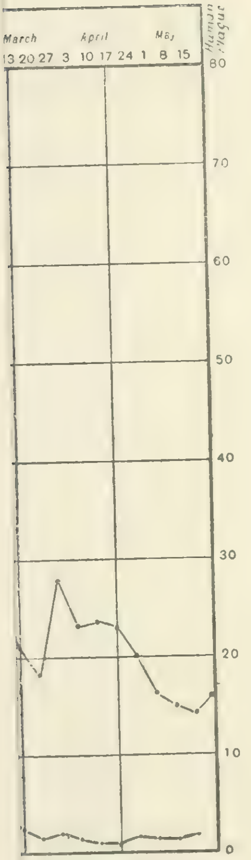
A similar seasonal variation in the prevalence of *X. cheopis* and correspondence between the maximum of these fleas and the epidemic period has been observed by Kitasato (1909) in Japan, Tidswell (1910) in Sydney, and by Gauthier and Raybaud (1910–11) at Marseilles, and by Andrew (1911) in Tongschang in Northern China. The incidence of the epidemic period in the season of greatest flea prevalence under such different climatic conditions as obtain in Bombay, the Punjab, Poona, Tongschang, and Marseilles, is in itself suggestive, and, in view of the results of animal experiments already referred to, one is justified in believing that the most favourable time for the epidemic is largely determined by the seasonal prevalence of rat-fleas.

(5) The rat-flea hypothesis also affords an interpretation of the fact that epidemics decline when the mean daily temperature passes 85° F. A mean temperature of 85° F. in India means also dry weather. Fleas, whether in the larval, pupal, or imago state, are rapidly killed off at this temperature unless associated with a high degree of humidity.

The Commission for the Investigation of Plague in India found that in Bombay the proportion of fleas which became infected, the time they remained infected, and the number of successful experiments on flea transmission was greatest during the winter months (Reports, 1907, pp. 197–204; 1908, pp. 510–515 and 529–538). An analysis of their results showed 67 per cent. successful transmissions when the temperature was 73° to 78° F., against 14 per cent. successes when the temperature was 82° to 85° F. Transmission experiments were therefore carried out simultaneously at 70° and 85° F. in suitable chambers both upon rats and guinea-pigs. The results were consistent with previous experience. The possibility that the virulence of the bacillus might undergo some variation if grown at one or other temperature was examined by the Commission and excluded.

The claims of flea transmission to be the predominating mechanism of spread from rat to man may be briefly summarized as follows:

1. The experimental evidence that plague is easily transmitted from animal to animal by rat-fleas.
2. That in presence of fleas, the epizootic, if started, varies as regards severity and rate of progress with the number of fleas present and the season of the year, whereas all attempts to induce epizootics in the absence of fleas have failed.
3. That under natural conditions (experiments in plague-houses, etc.) an animal can be protected from infection by any simple procedure which will exclude the visits of fleas.
4. The only discovered infection in plague-houses resides in plague-infected fleas.
5. Rat-fleas *X. cheopis* and *Ceratophyllus fasciatus* readily bite man.
6. The conclusions drawn from animal experiments, when applied to the problem of the spread of plague amongst human beings, afford a reasonable interpretation of every cardinal epidemiological fact.



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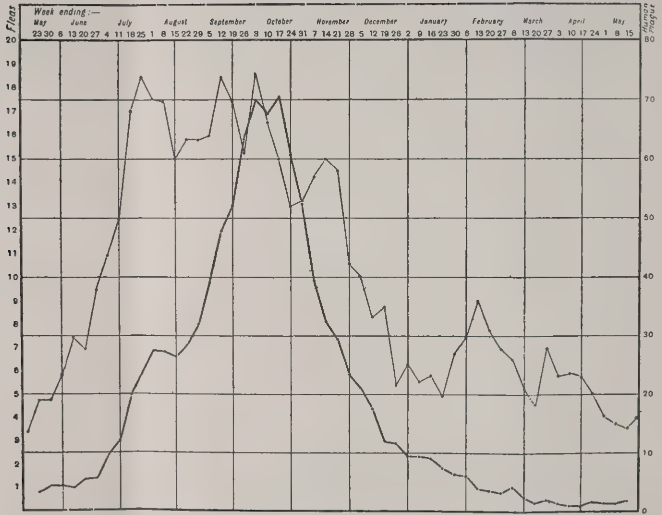

Fig. 17.—BELGAUM. ——— Average number of fleas per *Mus rattus*: weekly figures: observations made in 1908–1909. ——— Average number of deaths from plague, calculated for each week of the year, from May, 1897, to May, 1909 (twelve years), in Belgaum city.

The Part taken by the Human Flea.

It is possible to transmit plague experimentally by means of *P. irritans.* Nevertheless the direct transmission of the disease from man to man cannot, at the present time, be of frequent occurrence, or we should have evidence of direct infection instead of dependence upon the epizootic.

The reason why the human flea is ineffective is because in human cases the average degree of septicaemia before death is so much less than in rats that the chance of a flea imbibing even a single bacillus is small.

A variation of the plague bacillus in the direction of greater infectivity, with perhaps diminished toxicity leading to a higher degree of septicaemia in man, would permit of direct transmission by human fleas. Bubonic plague would then be independent of the rat, and spread directly from man to man. For several reasons it seems to me not improbable that this may have happened in the plague of the Middle Ages.

SOME OTHER DISEASES TRANSMITTED BY INSECTS.

Three other diseases which can be transmitted by insects—typhus, relapsing fever, and poliomyelitis—must be mentioned, although I am not sure that I am justified in including any of them under the heading "bacterial." The infective agent in typhus has, so far, not been isolated, but it is ascertained that it is an organism capable of propagation, for Nicolle (1910) and Wilder (1911) have succeeded in carrying the disease through a number of generations of monkeys by inoculating them with 1 c.cm. to 5 c.cm. of blood or serum from an infected animal. The organism is held back by Berkefeld and porcelain filters. It is, therefore, free in the blood and of some considerable size.

Relapsing fever is caused by a spirochaete, and there is considerable difference of opinion as to whether spirochaetes should be classed amongst the bacteria or protozoa.

The infective agent of poliomyelitis is a minute invisible organism, which passes readily through a porcelain filter, so that it escapes the clutches of the systematist altogether.

THE TRANSMISSION OF TYPHUS FEVER BY LICE.

Typhus is a disease associated with poverty and dirtiness. It occurs in temperate climates, and mostly in the winter and spring. In warm climates it is uncommon. In Mexico, where it still flourishes amongst the poor Indians and half-caste population, it is confined to the central plateau 4,000 to 10,000 feet above the level of the sea, and does not affect the population of the lower hot country, although cases are frequently introduced from the higher lands.

Typhus has been shown to be capable of transmission by the agency of body lice (*Pediculus vestimenti*) by

Nicolle, Compte, and Conseil (1909), and by Ricketts and Wilder (Wilder, 1911).

THE BIONOMICS OF LICE AND THE MECHANISM BY MEANS OF WHICH THEY MIGHT CARRY INFECTION.

Life-History of the Body Louse.

The life-history of the body louse has recently been studied by Warburton (1909), to whom and to my colleague, Mr. Bacot, I am indebted for most of the following facts on the subject. Unlike fleas, lice have no grub stage. The eggs take one to five weeks or longer to incubate, according to temperature. The young louse when just hatched is a small edition of the parent, and sucks blood at the earliest opportunity. It is, however, not sexually mature; three moults occur, and in about fourteen days its growth is complete and the insect is sexually mature. The females lay 100–200 eggs. Lice, especially when young, are delicate creatures. They are voracious feeders, and must be fed at frequent intervals. They, as a rule, are unable to stand starvation for more than two days. They are very sensitive to heat and drying, and soon succumb at temperatures above 25° C. unless the air be kept nearly saturated with moisture. They live in clothing and bedding, and feed several times in the twenty-four hours if opportunity be given. The optimum condition for their development occurs in the case of those persons who seldom wash their clothing or change it for sleeping.

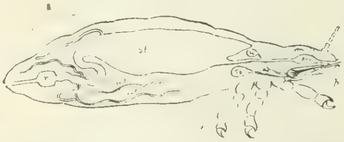

Fig. 18.—Alimentary canal and mouth parts of louse (*Pediculus vestimenti*).

The general arrangement of the alimentary canal may be seen in Fig. 18, which also shows the sac in the floor of the mouth which contains the piercing organ. The four salivary ducts (two only of which are shown) open into the base of this sac. The pharynx is a chitinous organ which collapses by its elasticity, and suction is produced by the contraction of the muscles (*m*) attached to its dorsal surface and the chitinous skeleton of the cranium. The blood is forced into the stomach by the relaxation of these muscles in order from before backwards.

The stomach or crop is a capacious organ with two later diverticuli from its anterior end.

The body louse obtains its nourishment entirely by sucking blood. It is provided with penetrating instruments the apposition of which forms a canal through which the blood is pumped into the stomach by a pharyngeal pump similar to that of the flea. These piercing instruments are not permanently exterior to the head and carried tucked away in a labium as in fleas and bugs, but, as described by Pawlovsky (1906), the apparatus is retractile and contained in a special pocket in the floor of the mouth (Fig. 18, *p*). The anterior part of the mouth is provided with a ring of hooklets, which is everted when the pricker is thrust out (Fig. 19 A, *h*).

When the insect feeds, the tubular "pricker" (*p*) is protruded through the mouth and penetrates the skin of the host, and the hooklets serve to attach the mouth to the skin. The exact way in which the sucking tube is built up is not quite clear, but from preparations made by my colleagues Mr. Bacot and Dr. Rowland* the arrangement of these parts appears to be as follows.

Three elements enter into the formation of the "pricker," each of which is bifurcated at its base (see Fig. 19 A). From the dorsal and ventral aspects of two of these parts, just anterior to their bifurcation, a chitinous expansion is given off which runs forward and embraces the finer piercing organs (Fig. 19 A and Fig. 19 B, *Ch*). The two together form a sheath round the basal portions of the piercing organs.

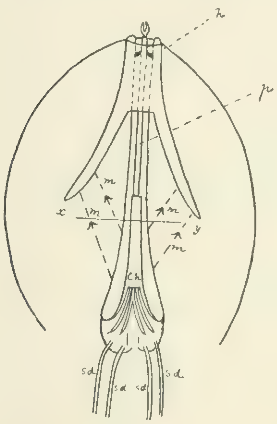

Fig. 19 A^{1}.

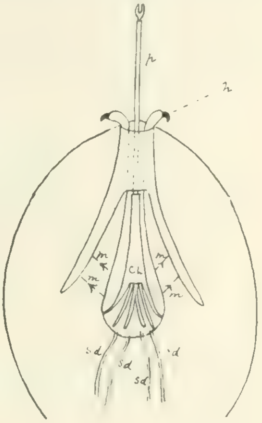

Fig. 19 A^{2}.

The relation of the three elements to one another and how the sucking tube is formed may be seen from Fig. 19 B, which represents a transverse section in the neighbourhood of the line *x-y* in Fig. 19 A^{1}; *Ch* is the chitinous sheath, *a* the dorsal element which we may call the stylet, *b* the median, and *c* the ventral element.

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* I am greatly indebted to Mr. Bacot and Dr. Rowland not only for lending me specimens of the mouth parts of the louse, but also for their kindness in making fresh dissections for the purpose of this lecture. The mechanism of the sucking apparatus of the louse is much more difficult to comprehend than that of the flea or bug, and the description given merely represents the conclusions I have been able to arrive at without a prolonged study of the intricate arrangement of the organ.

The interlocking of the lateral chitinous expansion of *b* and *c* forms the canal *s.t.* up which blood is sucked.

Fig. 19 A¹, is a diagrammatic representation of the head of a louse with the pricker retracted, A² with the pricker extruded; *p* is the "pricker" made up by the apposition of the three parts mentioned above. Anteriorly these pass through a tunnel in the substantial chitinous piece shaped like an inverted Y, which apparently also forms a skeletal framework for the attachment of the muscles (*m*, Fig. 19 A) which pull the whole organ forward.

Ch is the sheath covering the basal portion of the piercing parts. At the base the bifurcation of the three elements is seen, and also the four salivary ducts (*sd*) which enter there, but the precise connexion of which has not yet been made out.

When the whole organ is pulled forwards it not only drives out the "pricker," but evaginates the lining membrane of the sac and everts the ring of hooklets (*h*) whereby the head is anchored to the epidermis.

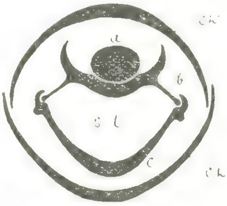

Fig. 19 B.

Summary of the Position with regard to the Transmission of Typhus by Lice.

1. The infection of typhus circulates in the blood during and for some time after the febrile period.
2. It is taken up by lice when they feed upon a patient, and may be transmitted to monkeys by them.
3. As in the case of fleas and plague, a considerable number of insects—ten or more—are required to give a reasonable chance of infection to a monkey, an animal which is probably much less sensitive than man.
4. There is reason to believe that the virus proliferates inside the insect; for, in order to produce infection by means of blood taken from a patient, the volume required is much larger than could possibly be contained in the lice employed (Wilder).
5. The infectivity of lice endures seven days, and seems to be greater some days after feeding on a typhus patient than immediately (Nicolle and Wilder), but the number of experiments so far performed is not adequate to establish this.
6. There is reason to believe that the infection may be transmissible to a second generation of lice (Wilder).

7. The present limitation of outbreaks of typhus to the most vermin-infested section of the population, its occurrence at a period of the year when the clothing is rarely removed from such persons, and washing of either takes place at considerable intervals, and the practical limitation of the disease to cold climates, all receive ready interpretation on the assumption that lice are essentially the porters of infection.

8. Further support is provided by the non-infectivity of typhus under modern hospital conditions, and the success attending prophylaxis directed towards the extermination of insect parasites: and I am unaware of any epidemiological fact inconsistent with the view that lice carry the infection.

The Transmission of African Relapsing Fever by the Tick.

Relapsing fever is widely distributed about the world, and was at one time very common in this country. In 1868 Obermeier discovered the presence of spirochaetes in great abundance in the blood of patients during the febrile attacks. These were subsequently shown to be the cause of the disease.

In 1905 Dutton and Todd (1905), in the Congo, and Koch (1905) in German East Africa, discovered that the tick-fever of Africa was relapsing fever, and that the spirochaete was conveyed from patient to patient by the tick *Ornithodoros moubata*.

Bionomics of Ornithodoros moubata.

These ticks are common in the native caravan routes. The adult feeds several times and lives a long time. They are very hardy insects, and can endure many months without food both in the larval and adult stages. In their habits they more resemble the bed-bug than the majority of the Ixodes; during the day they hide in the earth of the floors or in crevices in the walls and roofs, and at night emerge to feed upon the occupants. The female lays several broods of some hundreds of eggs in crevices of the ground. Incubation lasts from eight days to two months, according to temperature. The first moult occurs within the egg. The young larval *Ornithodoros* somewhat resembles its parent, has four legs but undeveloped sexual organs. It feeds upon blood and moults a few times, after which it is a sexually mature insect.

The number of infected ticks along the caravan routes of German East Africa is considerable. Koch (1905, 1906) found 5 to 15 per cent. contained spirochaetes, and in one instance 50 per cent. of the ticks collected from a particular locality contained spirochaetes.

Ticks, once infected, may retain the infection and transmit it to their progeny, for Dutton and Todd (1905) and Koch (1905–06) found that young ticks raised from the egg were infective. Möllers (1908) further discovered that the second generation from infected ticks were also capable of giving rise to relapsing fever. The same fact was demonstrated by Hindle (1912) for *Spirochaeta gallinarum* and *Argas persicus*, the fowl tick. These facts, no doubt, account for the large proportion which harbour the infection.

The precise mechanism of transmission in relapsing fever is still uncertain. For a few days after feeding on an infected animal Dutton and Todd (1905) and Koch (1905) found the spirochaetes in the alimentary canal of the tick. Subsequently they disappeared from the stomach

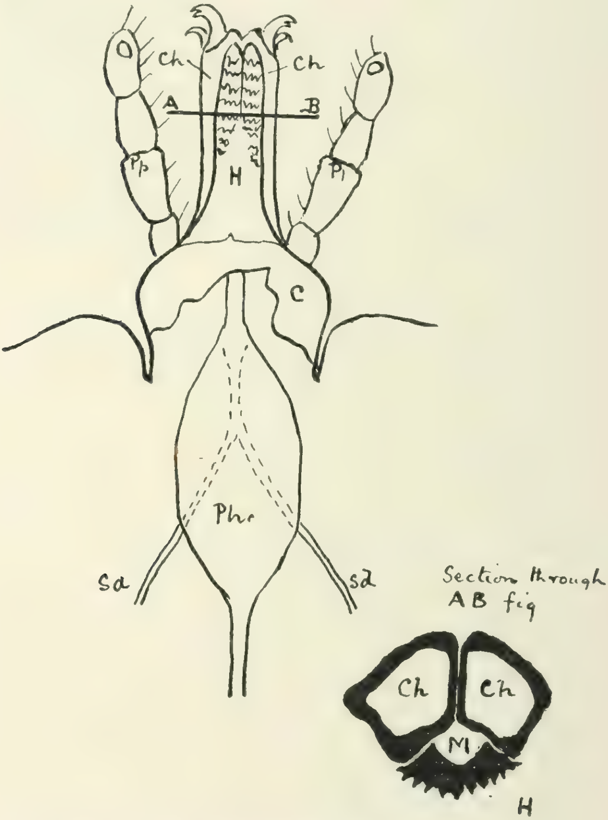

Fig. 20.—Mouth parts of tick (*Ornithodoros moubata*).

and gut, but were found by these observers in some of the internal organs, ovaries, Malpighian tubes, and salivary glands.

Leishman (1909), working in London upon a number of ticks sent from Africa, failed to find spirochaetes, although the ticks were capable of infecting monkeys. He noticed, however, that the cells lining the Malpighian tubes contained large numbers of minute chromatin-containing granules, and that similar granules were present in the ovary, and eggs of ticks, which subsequently gave rise to infective individuals. Ticks in which he failed to find these granules did not give rise to infection.

From these and other observations Leishman was led to the conclusion that the chromatin granules represent a stage in the development of the parasite. These interesting observations have been confirmed by Balfour (1911) in Khartoum in the analogous case of *Argas persicus* and fowl spirochaetes. Some experiments by Hindle (1911) also have a bearing upon this point. Hindle failed to find any spirochaetes in infected ticks kept some time at 21° C., but similar ticks warmed up to 35° C. for a few days showed spirochaetes throughout the body, and all the organs were infective, when injected into suitable animals.

The Structure and Arrangement of the Mouth Parts and Alimentary Canal of Ornithodoros moubata.

The general arrangement of the mouth will be seen from the diagram Fig. 20. The boring instrument or rostrum consists of three parts, two chelicerae (*ch*) and a ventral hypostome (H). The chelicerae are hollow chitinous organs inside which are muscles which operate the terminal claws; the hypostome is barbed. In the act of boring the claws are straightened and the three parts of which tha rostrum is composed are advanced a little further. The claws are again advanced for a further pull, and the barbed hypostome prevents any slipping back. By a succession of these efforts the rostrum is buried in the skin.

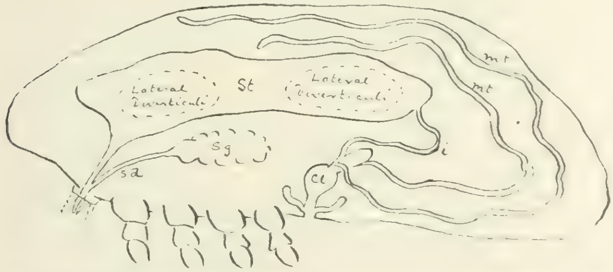

Fig. 21.—Alimentary canal and mouth parts of tick (*Ornithodoros moubata*).

The three instruments of the rostrum, when apposed, form a canal, at the base of which is the mouth. This widens into a pharynx, which is alternately compressed and expanded by a co-ordinated contraction of the individual muscle fibres attached to it. The blood is driven by the pump through the narrow oesophagus into the crop or stomach (*st*). The stomach is a capacious pouch (Fig. 21), having extensive lateral diverticula, which themselves branch so that after a full meal the organ distends the insect. The intestine is a very narrow tube connecting the stomach to the cloaca (*c*). The anus is not terminal, but opens in the mid-line just behind the last pair of legs.

The salivary glands (*sg*) are of the compound racemose type, each with one duct (*sd*) passing forward to the mouth.

Koch was of opinion that the spirochaetes passed from the salivary glands during the act of sucking, but both Leishman and Hindle consider that the infection is not transmitted by this channel, but that the infective excrement from the Malpighian glands passes out per rectum, and is washed into the puncture made by the insect by means of the coxal secretion which is deposited during the latter stages of feeding.

THE TRANSMISSION OF THE RELAPSING FEVERS OF EUROPE, AMERICA, AND INDIA.

Whether the spirochaetes which are responsible for relapsing fever in various parts of the world represent distinct species (Novy and Knapp, 1906; Uhlenhuth and Haendel, 1907), or merely varieties, does not seem to me proven in view of the observations of Darling (1909), and Manteufel (1908). Anyhow, they are very similar, and can all be transmitted by the tick *Ornithodoros moubata* (Manteufel, 1908, and Neumann, 1909). It is, however, clear that this tick was not the transmitting agent in Ireland and England, where relapsing fever was common fifty years ago, nor is it so in Russia, America, or India, at the present time.

The epidemiology of the disease in these countries is not yet clear. Spirochaetes are such obligatory parasites that the agency of an insect transmitter seems almost necessary. The only certain way of producing the disease is by inoculating a minute quantity of the blood of a patient during the febrile stage. Spirochaetes have been found in the urine, but no one has succeeded in producing infection by this means. It must also be remembered that they can find their way through the unbroken skin.

The distribution of relapsing fever among the population is consistent with the view that insects are necessary, for, like typhus, this disease is in Europe almost confined to the poorest and dirtiest members of the community. It has been frequently suggested that bugs might disseminate the disease. Tictin (1897) and Karlinski (1902) found that the stomach contents of the bed-bug remained infective for two or three days after feeding on the blood of a patient, and Kladnitzky (1908) observed appearances strongly suggestive of multiplication of the spirochaetes in the stomach of the bug. With one exception, however, all the numerous attempts to transmit the disease by the bed-bug have been unsuccessful. Experiments have also been made with fleas by Sergent and Foley (1908) and others. The results were negative. A further reason for doubting the importance of either of these insects is that the disease is not more prevalent at the time of year these insects abound, but rather the contrary. In Europe the disease occurred in epidemic form throughout the year, the greatest prevalence being usually during winter and spring.

The only remaining ectoparasites are lice—*Pediculus capitis* and *Pediculus vestimenti.* The general bionomics of these insects has been dealt with, and it only remains to point out that their prevalence is not confined to the warmer months, but is greatest during the winter (Hamer, 1910), when clothes are least changed and washed.

Mackie (1907), in an investigation of an outbreak of relapsing fever in a mission school in India, brought forward some rather striking epidemiological facts pointing to lice, rather than bugs, as being concerned with the epidemic. He found spirochaetes in 14 per cent. of the lice taken off the boys. They were present in the alimentary canal, ovary, and Malpighian tubes of these insects. From his observations he came to the conclusion that spirochaetes multiplied greatly in the stomach of the insect, reaching their maximum on the third day. Mackie fed lice from the patients upon monkeys, but he failed to transmit the disease. In two experiments by Sergent and Foley (1910) relapsing fever followed the infection of the clothing by lice from patients.

The spirochaetes of relapsing fever infect rats, and Manteufel (1908) made some observations on the capacity of the rat-louse (*Haematopinus*) to transmit the disease. He first ascertained by forty experiments that, when free from lice, sick and healthy rats could be caged together without the latter contracting the disease, and then repeated the experiments with the addition of lice. Two infected rats were caged together with seven heathy ones. The sick animals were killed by ether at the height of the disease, and then put back into the cage in order that the lice might crawl off the cadavers and attach themselves to the living animals. Three of the seven became infected. In another similar experiment with *Spirochaeta duttoni*, one out of eight rats became infected. Manteufel could not find spirochaetes in the louse outside the alimentary canal, nor in the eggs.

These experiments were confirmed by Neumann (1909), so that it seems highly probable that relapsing fever is spread by lice as well as by the tick *Ornithodoros moubata.* Although the former agent may not be nearly so efficient as the latter, it may make up by its greater numbers and persistency.

The details of transmission by lice are still unknown, but, from the habits of these insects to freely discharge the contents of their alimentary canal during feeding, and the readiness with which spirochaetes can penetrate uninjured epithelium, it is not difficult to imagine how this may happen, even supposing the salivary secretion does not contain the infection.

Transmission of Poliomyelitis by Stomoxys calcitrans.

At the International Congress on Hygiene, held in Washington last September, Rosenau announced that he had succeeded in conveying poliomyelitis from an infected monkey to several other monkeys by means of the bites of *Stomoxys calcitrans*, the small biting stable-fly.

This observation has recently been confirmed by Anderson and Frost (1912). Three monkeys exposed daily to the bites of 300 *Stomoxys* which had previously fed on two infected monkeys during the whole course of their illness all developed the disease seven to nine days after their first exposure. The observation is of particular interest because hitherto the transmission of poliomyelitis by the subcutaneous inoculation of blood from an infected monkey has been very uncertain even when large quantities of blood have been transferred. It suggests that either the previous experiments have been made at the wrong stage of the disease, or else that the virus multiplies or intensifies in the body of the fly. The seasonal incidence of the disease being summer and autumn is consistent with insect transmission, but the continuance of the epidemic in Sweden after flies have disappeared indicates that there are other means of spread.

BED-BUGS (CIMEX LECTULARIUS) AS PORTERS OF INFECTION.

The general career of the bed-bug is more or less familiar to most people, but it may not be unnecessary to point out that, unlike fleas, there are no larval or pupal stages, but the insect emerges from the egg as a little bug. For the following facts I am indebted to my colleague, Mr. Bacot, who has made observations on this subject.

The young bug sucks blood on the earliest opportunity, grows and moults, and feeds again. After five moults it is a sexually mature and adult insect. Bugs only visit their hosts for food, and then preferably at night; they strongly resent light, and during the day hide themselves in some cranny of the room or furniture. They are very hardy and do not require the constant feeding which is so troublesome in breeding lice and fleas. They can survive without food for six months or more.

A bug which has imbibed a good meal retires to some secluded spot and slowly digests it. It is some days before he develops a fresh appetite and sallies forth again in search of prey.

The Arrangement of the Alimentary Canal and Mouth Parts of the Bed-Bug.

The general scheme of the alimentary canal will be sufficiently clear from the diagram (Fig. 22). There are two pairs of salivary glands (*sg* and *sg*[1]) and the duct of the larger of the dorsal pair (*sg*[1]) bifurcates shortly after it emerges from the gland and both tubes run forward to the base of the piercing organ. The exact way in which the three salivary ducts on each side terminate has not yet been determined. The mouth parts also differ from those of the flea in that the sucking tube is made up by the apposition of four elements instead of three. These are two maxillae (*mx*) and two mandibles (*mn*). When not in use these delicate piercing instruments are carried in a groove on the dorsal aspect of the three-jointed labium (*la*). Whilst feeding this support is folded backwards under the head.

Experiments on the Transmission of Relapsing Fever, Typhus, and Plague by Means of Bed-Bugs.

The observations of Tictin have already been referred to. Although these show that the spirochaetes of relapsing fever may live from some hours to many days, according to circumstances, in the stomach of the insect, they give no information as to whether the bed-bug can in a natural way transmit the disease.

Christy (1902) and M. Rabinowitch (1907) and Shellack (1909) tried the experiment on themselves without success. Their attempts were very thorough; Shellack, for instance, fed 168 bugs, which had been previously nourished on a diet of rat-blood infected with *Spirochaeta recurrentis*, upon himself. The experiment lasted a month, and was so arranged that a variety of intervals elapsed between the ingestion of infected blood and the second meal.

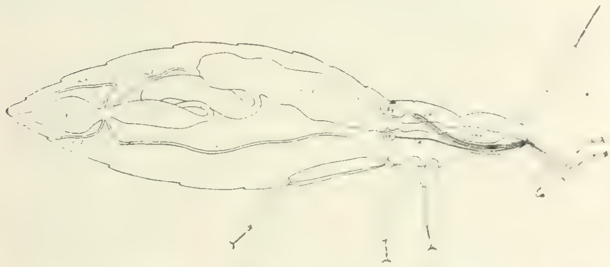

Fig. 22.—Alimentary canal and mouth parts of bug (*Cimex lectularius*).

Breinl, Kinghorn and Todd (1906), and Rabinowitch (1907), and Sergent and Foley (1910) have also tried to transmit relapsing fever to monkeys by means of bugs. Large numbers of insects were employed by the former observers, sometimes as many as 590 to one monkey.

Their experiments were carried on over two or three months. Bugs which had fed upon an infected monkey at all stages of the disease were used, and the interval between feeding upon the infected and healthy animal varied. Nevertheless, no transmission of the disease occurred.

The only case of a successful transmission was one experiment by Nuttall (1907), who removed a bug shortly after it had commenced to feed upon an infected mouse, and allowed it to complete its meal upon a normal mouse.

Bed-bugs were excluded from consideration as transmitters of typhus by Nicolle (1910) and by Wilder (1911), as the distribution of these insects both in time and space does not coincide with that of typhus. Wilder, however, tried experimentally to convey the disease from monkey to monkey by bugs, but without success.

Verjbitski (1904) made a number of experiments on the transmission of plague by the bed-bug. He showed that plague bacilli multiply in the stomach of bugs, as they do in that of fleas, and that their virulence was unimpaired. He succeeded in conveying plague to guinea-pigs by the bites of bugs, but only up to four days after they had fed on an infected animal.

There is really no evidence to incriminate the bed-bug in the case of either typhus or relapsing fever. It is possible to transmit plague experimentally by means of bugs, but there is no epidemiological reason for supposing this takes place to any extent in nature.

There are two differences in the habits of bugs and those of fleas and lice which may possess epidemiological significance. The first concerns their customary intervals between their meals. Bugs show no disposition to feed for a day or two after a full meal, whereas fleas and lice will suck blood several times during the twenty-four hours. The second is in respect to the time the insects retain a meal and the extent to which it is digested before being excreted. Fleas and lice, if constantly fed, freely empty their alimentary canals, and the nature of their faeces indicates that the blood has undergone but little digestion.

Both these insects evacuate such undigested, or half digested, blood per rectum during the act of feeding, and the remnants of the previous meal are thus deposited in the immediate vicinity of a fresh puncture. It is not unlikely that, should the alimentary canal of the insect be infected with plague bacilli, spirochaetes, or the organism responsible for typhus fever, these may be inoculated by rubbing or scratching.

Bugs have not this habit, and in all the cases I have examined their dejections were fully digested, almost free from protein, and consisted mostly of alkaline haematin.

This is, however, probably not the whole explanation, and there is, I believe, much yet to learn as to the mechanisms of insect transmission, and why one blood-sucking parasite is an effective porter and another not, even in the cases of bacterial infection where no stage in the life-cycles of the organism takes place within the insect host.

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AN EXPERIMENT TO ILLUSTRATE THE EFFECT OF SIZE OF POPULATION ON THE RATE OF SELECTION OF NEW BACTERIAL RACES.

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IN the course of a research into the question of the selection of a variant of *B. typhosus* in respect of dulcite-fermenting power, the following experiment was designed with the view of ascertaining whether the bulk of the fluid medium in which the organism was grown had any marked influence on the rate of selection of the new race.

A set of nine beakers, each beaker being 5½ cm. in diameter, and a set of nine test tubes, each tube being 1 cm. in diameter, were filled to the depth of 1 in. with the same sample of dulcite peptone water of the following composition, and containing litmus as indicator :

Peptone (Witte)	20 grams
Dulcite	5 ,,
Salt	5 ,,
Water	1 litre

The dulcite-peptone water was rendered faintly alkaline to litmus and sterilized by steaming on three successive days. The tubes and beakers were of the same height, so that the condition of aëration of the medium was strictly similar in each series. This precaution is necessary, as it is well known that alkali formation from the peptone is greatly favoured by free access of air. Each member of these two series was inoculated with the same quantity of *B. typhosus* broth. The strain selected for this purpose was one which, from previous testing, was known to acquire the power of fermenting dulcite more slowly than the majority of strains. All the cultures were incubated at 37° C.

The time taken to produce acidity in the original beakers and tubes is given in Table I, and it will be noted that no test-tube culture became acid during the period of observation (twenty-six days).

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After eight days' growth subcultures were made from each tube and each beaker into 6 c.cm. quantities of dulcite-peptone water contained in ordinary test tubes. The times taken to produce acidity in these subcultures are given in Table II. Again no subculture from the original test tubes became acid.

After a further period of seven days—that is, fifteen days from the commencement of the experiment—subcultures were again made from the original beakers and tubes into ordinary 6 c.cm. quantities of dulcite broth. The times required for appearance of acidity are indicated in Table III.

These various tables show that a fermenting race is much more quickly produced by growth of the organism in large quantities of culture medium than in small quantities. If the original small tubes be inoculated with small doses of *B. typhosus* broth and the beakers with large doses, the sizes of these doses being related to each other as the volumes of the dulcite broths to be inoculated, the fermenting race is again found to arise more quickly in the large quantities.

The original small tubes never developed an acid reaction, and their subcultures likewise did not, within the limits of observation. The large cultures in beakers developed an acid reaction in every case by the fifteenth day. Their first subcultures showed three with a full acid reaction by the tenth day. In the second subcultures from the original beakers nine became fully acid in reaction by the sixth day.

The experiment above detailed was led up to by a preliminary experiment performed with a *B. typhosus* strain which was known to acquire the power of fermenting dulcite more quickly than the above-mentioned strain. The details of this earlier experiment were as follows: 50 c.cm., 20 c.cm., and 5 c.cm. quantities of dulcite-peptone water were put up in three series. In each series there were ten such quantities. Every member of the different series was inoculated with the same dose of *B. typhosus* broth. On the tenth day after inoculation all the broths were subcultured into ordinary test tubes containing 6 c.cm. quantities of dulcite broth, and the times required to produce acidity were noted. The results are given in Table IV.

It will be observed that all the subcultures from the initial cultures of 50 c.cm. showed full acidity by the second day. The subcultures from the 20 c.cm. quantities showed four with a full acid reaction on the second day. In the case of the subcultures from the 5 c.cm. tubes not one showed a full acid reaction on the same day.

We conclude, therefore, that, other things being equal, the larger the culture the sooner will a new race be selected out. This more effective selection from a large population is mainly due to there being more extreme variants from the mean in the large population. The explanation is not chiefly to be sought in the fact

TABLE I.

Days of Observation.

Beakers.

Days of Observation	1	2	3	4	5	6	7	8	9	CONTROL
8th	—	—	—	—	—	—	—	—	—	—
9th	—	—	—	—	—	—	—	af	—	—
12th	—	—	—	—	—	—	—		—	—
13th	af	—	$a\frac{1}{3}$	—	—	—	—		—	—
14th		$a\frac{1}{2}$	af	$a\frac{1}{2}$	—	$a\frac{1}{2}$	—		$a\frac{1}{2}$	—
15th		af		af	af	af	af		af	—

Small Tubes.

Days of Observation	1	2	3	4	5	6	7	8	9	CONTROL
8th	—	—	—	—	—	—	—	—	—	—
9th	—	—	—	—	—	—	—	—	—	—
13th	—	—	—	—	—	—	—	—	—	—
14th	—	—	—	—	—	—	—	—	—	—
15th	—	—	—	—	—	—	—	—	—	—
20th	—	—	—	—	—	—	—	—	—	—
26th	—	—	—	—	—	—	—	—	—	—

TABLE II.

First Subcultures from Beakers into Small Tubes (6 c.cm.).

Days of Observation.	1	2	3	4	5	6	7	8	9	CONTROL
2nd	–	–	–	–	–	–	–	–	–	–
6th	–	–	–	–	–	–	–	–	–	–
7th	–	–	–	–	–	–	–	af	–	–
8th	–	–	–	–	–	–	–		–	–
10th	af	–	af	–	–	–	–		–	–
11th		–		–	–	–	–	–	–	–
19th		–		–	–	–	–	–	–	–

First Subcultures from Small Tubes into Small Tubes (6 c.cm.).

Days of Observation.	1	2	3	4	5	6	7	8	9	CONTROL
2nd	–	–	–	–	–	–	–	–	–	–
6th	–	–	–	–	–	–	–	–	–	–
7th	–	–	–	–	–	–	–	–	–	–
8th	–	–	–	–	–	–	–	–	–	–
10th	–	–	–	–	–	–	–	–	–	–
11th	–	–	–	–	–	–	–	–	–	–
19th	–	–	–	–	–	–	–	–	–	–

TABLE III.

Days of Observation.

Second Subcultures from Beakers into Small Tubes (6 c.cm.).

1	2	3	4	5	6	7	8	9	CONTROL	
$a\frac{1}{4}$	–	$a\frac{3}{4}$		–	–	–	$a\frac{1}{4}$	$a\frac{1}{4}$	–	2nd
af	–	af	$a\frac{1}{2}$	$a\frac{1}{2}$	$a\frac{1}{2}$	–	$a\frac{3}{4}$	$a\frac{3}{4}$	–	3rd
	–		af	af	af	–	af	af	–	4th
	$a\frac{1}{2}$					$a\frac{1}{2}$			–	5th
	af					af			–	6th

Second Subcultures from Small Tubes into Small Tubes (6 c.cm.).

	1	2	3	4	5	6	7	8	9	CONTROL
2nd	–	–	–	–	–	–	–	–	–	–
4th	–	–	–	–	–	–	–	–	–	–
6th	–	–	–	–	–	–	–	–	–	–
11th	–	–	–	–	–	–	–	–	–	–

Af = full acid reaction. *A* = acid. The fractions indicate grades of partial acidity.

TABLE IV.

	1	2	3	4	5	6	7	8	9	10	Day of Observation.
Subcultures from tubes of 50 c.cm.	A.f.	A.f.	A.f.	A.f.	A.f.	A.f.	A.f.	A.f.	A.f.	A.f.	Second day.
Subcultures from tubes of 20 c.cm.	A.f.	A.½	A.f.	A.½	A.½	A.½	A.f.	A.½	A.f.	A.½	Second day.
,, ,,		,,		,,	,,	,,		,,		A.f.	Fourth day.
,, ,,		,,		,,	,,	,,		A.¾			Fifth day.
Subcultures from tubes of 5 c.cm.	A.¼	A.¼	A.¼	A.¼	A.¾	A.¼	A.¼	A.¼	A.¼	A.¼	Second day.
,, ,,	A.½	,,	,,	,,	A.f.	,,	,,	,,	,,	A.¾	Fourth day.
,, ,,	A.f.	A.½	A.½	A.½		A.½	A.½	A.½	A.½	A.f.	Fifth day.

that in the larger cultures a few more generations are required to produce the same population per cubic centimetre. By inoculating into constant volumes of dulcite broth seedings of *B. typhosus* of different size the number of generations required to produce the same population per cubic centimetre is altered, but the tubes with small seedings do not give regularly earlier acidity than the tubes with large seedings.[1]

Since bacterial selection can be conducted more efficiently from a large population, it would appear probable that the selection of variants by animal passage would, other things being equal, be more successful in a large rather than in a small animal.

CONCLUSION.

The rate of selection of dulcite variants of *B. typhosus* varies directly with the size of the population from which the selection is made.

REFERENCE.

[1] Penfold (1911): Studies in Bacterial Variation, *Journal of Hygiene*, vol. xi, p. 30.

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THE INHIBITORY SELECTIVE ACTION ON BACTERIA OF BODIES RELATED TO MONOCHLORACETIC ACID.

(A CONTRIBUTION TO THE THEORY OF CELL INTOXICATION.)

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(With Plate II.)

THE author (1911) showed that growth of *B. coli* (Escherich), on monochloracetic acid agar, led to the selection of a new strain unable to produce gas from glucose, lactose and other sugars, but still able to produce gas from alcohols in very considerable amount. Harden and Penfold (1912) compared the chemical action of this new strain with that of the original strain when grown anaerobically on glucose peptone water. The general result of the examination was to show that the new strain produced much more lactic acid, and less alcohol, acetic acid and formic acid, the gas being reckoned as formic acid, than the original strain. This pointed to the fact that the lactic acid-producing ferment was distinct from that ferment which produced alcohol, acetic acid and formic acid. Harden (1901) had previously brought forward evidence showing that the CO_2 and H were derived from the splitting up of formic acid. Since the new strain which could not produce gas from glucose, produced a normal yield from sodium formate, Harden and Penfold concluded that the splitting up of the formate was due to a third enzyme. This fermentation of glucose is shown schematically below:

Glucose — Lactic acid, by enzyme I.
— Acetic acid, Alcohol, Formic acid } by enzyme II.

Formic acid — Hydrogen, Carbonic acid gas } by enzyme III.

Six different strains of coliform organisms, and also *B. enteritidis* Gaertner, *B. paratyphosus* B. and *B. Grünthal*, were found constantly to give non-gas-producing strains when grown on chloracetate agar and tested in glucose. It was no accidental variation, for the new strains could be produced with certainty in ten days.

In the endeavour to understand the above selection process, various organic substances were added to agar and the evidences of variation

TABLE I.

The cultural signs of variation and selection obtained when B. coli *(Escherich) is grown on phenylacetic acid agar plates.*

No. of plate	Quantity of 10 % solution of phenylacetic acid added to respective plates	Days of observation		
		2nd	4th	7th
1	None	Good growth. No variability in the size of the colonies. No secondary colonies present.	No secondary colonies. No variability in the size of the colonies other than that due to crowding.	As last observed.
2	None	,,	,,	,,
3	0·05 c.c.	,,	Definite naked eye papillae present on the colonies.	Many large papillae present on colonies. Marked variability in size of colonies.
4	0·3 c.c.	,,	,,	,,
5	0·5 c.c.	,,	,,	,,
6	0·7 c.c.	,,	,, Some colonies show swollen margins.	,,
7	1·0 c.c.	,,	Papillae present on some colonies. Big and little colonies are present in central crowded portions of plate.	,,
8	1·3 c.c.	,,	,,	,,
9	1·6 c.c.	,,	,,	,,
10	2·0 c.c.	,,	,,	,,
11	2·4 c.c.	Colonies inhibited in growth a little.	,,	,,
12	2·8 c.c.	Colonies inhibited in growth markedly.	As last observed.	,,

Remark. In the higher concentrations many of the colonies of large size are thickened and of a marked yellow colour.

and selection which had been obtained with chloracetate sought for. Some of these results are detailed in previous papers by the author.

It was found *e.g.* that monochloracetate of soda was much more inhibitory than dichloracetate or trichloracetate. Neither the dichloracetate nor trichloracetate produced the papillae on the surface of the plate colonies which are so characteristic of the monochloracetate Great variability in the size of the colonies—a feature which was strongly marked in the case of the monochloracetate plate cultures—did not occur.

TABLE II.

B. coli after growth on phenylacetic acid agar.

Agar slopes, from colonies of phenylacetic acid agar plates described in Table I, were inoculated in glucose and mannite peptone waters with the following result.

Variety of colony from which the agar slope was taken	Day of observation	Glucose	Mannite
From plate 10 of the series.			
(1) Large yellow colony	1	Af. G. 1/6	Af. G. 4/10
	2	Af. G. 1/5	Af. G. 5/6
(2) ,,	1	Af. G. 1/3	Af. G. 4/10
	2	Af. G. 5/12	Af. G. 2/3
(3) ,,	1	Af. G. 1/5	Af. G. 1/4
	2	Af. G. 5/12	Af. G. 3/4
(4) ,,	1	Af. G. 1/3	Af. G. 1/2
	2	Af. G. 5/12	Af. G. 5/6
(5) ,,	1	Af. G. 1/6	Af. G. 1/3
	2	Af. G. 1/3	Af. G. 2/3
(6) Little colony	1	Af. G. 1/6	Af. G. 1/2
	2	Af. G. 1/3	Af. G. 2/3
(7) ,,	1	Af. G. 1/4	Af. G. 7/16
	2	Af. G. 1/3	Af. G. 2/3
(8) ,,	1	Af. G. 1/4	Af. G. 7/12
	2	Af. G. 1/3	Af. G. 2/3
From plate 11 of the series.			
(A) Large colony	1	Af. G. 1/3	Af. G. 7/12
	2	Af. G. 5/12	Af. G. 7/12
(B) ,,	1	Af. G. 1/5	A. 3/4 G. 7/16
	2	Af. G. 1/3	Af. G. 5/6
(C) ,,	1	Af. G. 1/4	Af. G. 1/3
	2	Af. G. 1/3	Af. G. 2/3
(D) ,,	1	Af. G. 1/4	Af. G. 1/5
	2	Af. G. 1/3	Af. G. 1/2
(E) ,,	1	Af. G. 3/8	Af. G. 1/3
	2	Af. G. 1/2	Af. G. 3/4
From plate 12 of the series.			
(F) Large yellow colony	1	Af. G. 1/3	Af. G. 1/2
	2	Af. G. 5/12	Af. G. 2/3
(G) ,,	1	Af. G. 3/8	Af. G. 1/2
	2	Af. G. 5/12	Af. G. 3/4
(H) ,,	1	Af. G. 1/2	Af. G. 7/16
	2	Af. G. 7/12	Af. G. 3/4

Monobromacetate of soda added to agar gave the same papillae as the monochloracetate but big colonies did not appear in high concentrations as in the case of monochloracetate plates, and from none of the plates with lower concentrations, was it possible to obtain non-gas-producing strains.

Phenylacetic acid. Phenylacetic acid was dissolved in saturated sodium carbonate solution until the solution was just faintly alkaline to neutral litmus paper. Distilled water was then added to render the solution of 10 % strength. The solution was sterilized by filtration through a Doulton filter. Ascending quantities of the solution were put into Petri dishes and 15 c.c. of melted agar were added to each plate, the whole being then well mixed.

The plates were inoculated with *B. coli* (Escherich) and grown at 37° C. Their appearances are indicated in Table I.

The results recorded in Table I are similar to those obtained when *B. coli* (Escherich) was grown on chloracetic acid agar.

From plates 10, 11 and 12 of the series, after eight days' growth at 37° C., various colonies were inoculated on to agar slopes and subsequently tested on mannite and glucose peptone waters. The results are recorded in Table II[1].

It will be noticed that the average yield of gas of the strains from big colonies of plate 10 was the same as that from the little colonies, so that the phenylacetic acid does not appear to differentiate between the colonies in respect of gas-forming power. The gas-producing function in all the colonies is, however, slightly depressed.

Other substances which were tried, were found ineffectual in producing marked selections on the plates or non-gas-producing variants, but details of the experiments are omitted here because they are elsewhere dealt with and because of the negative results. The substances in question were cyanoacetic acid, α-bromproprionic acid, dibromsuccinic acid, chlormalonic acid, hippuric and benzoic acid, all used as alkaline salts. On the other hand monochlorhydrin, a monochlor substitution product of glycerine, was found to give rise to a similar variant in the case of the *B. coli*, as monochloracetate of soda. The appearances indicating selection as they occurred in the colonies of the monochlorhydrin agar plates were somewhat different from those observed on monochloracetate of soda plates.

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[1] In Table II Af. = full acid reaction. G. = gas. The fraction after G. = the amount of the gas tube occupied with gas. The gas tests were performed in Durham's tubes. The fractions following A. indicate varying degrees of partial acidity, of the litmus solutions.

The *monochlorhydrin* medium is an agar to which a known quantity of 20 % filtered solution of monochlorhydrin is added. Ascending quantities of the solution were placed in Petri dishes and 15 c.c. of melted agar were added to each plate and thoroughly mixed. The plates were then inoculated with *B. coli* and grown at 37° C. The appearances obtained are given in Table III.

Table III shows, therefore, that at suitable concentrations marked variability in the size of similarly situated colonies may occur, and papillae may be present, which however are more inclined to be situated towards the cortex of the colonies than in the case of colonies of *B. coli* when grown on monochloracetate agar. The most peculiar feature of the plates is the large tree-like outgrowths from the periphery of the colonies. These seem to have some similarity to the appearances described by R. Müller (1909) in colonies of *B. paratyphosus* B. when grown on gelatine. (Pl. II, figs. 1, 2, 3.)

Subcultures on to agar slopes were made from different colonies of the series described in Table III and were then tested on carbohydrate media as recorded in Table IV.

The results of the examination of the different colonies and portions thereof show that the dense colonies and the papillae are good gas producers, whereas on chloracetate of soda plates the big dense colonies were either poor gas producers or failed to produce gas at all.

The lateral tree-like expansions of the colonies on the 0·2 c.c. chlorhydrin plates had, however, lost the power of producing gas from lactose. Two of these were tested on the whole series of carbohydrates with results given in Table V.

Table V shows that the variant *B. coli* obtained by growth on chlorhydrin agar is similar to the monochloracetate variety of the same organism. It gives either little or no gas from sugars and a fair yield from alcohols and it takes a longer time than normal to clot milk. On the other hand it appears almost entirely unable to produce gas from sodium formate while the normal strain gives 6/12 to 7/12 of a tubeful as also did the chloracetate variety of *B. coli* which I first described. It would be very interesting to know if an accumulation of formic acid is taking place in the glucose tubes on which this new variety is grown, but that point has not yet been decided.

TABLE III.

Record of plates of B. coli (*Escherich*) *on monochlorhydrin agar. Each plate contained* 15 *c.c. of agar and the quantity of* 20 % *chlorhydrin solution indicated.*

Amount of chlorhydrin solution	Day of observation			
	1st	2nd	5th	10th
None	Good growth.	Edges of colonies somewhat spreading in character.	As last observed.	No lateral expansions, no papillae, no variability in size of the colonies in centre of plate.
·1 c.c.	Slight inhibition of growth.	Edges of colonies sharper, but the colonies are well grown.	Lateral expansions present on all large colonies. Cortical papillae also present on colonies.	A few small opaque dense colonies outgrowing the rest in the crowded centre of plate. (See Pl. II, fig. 1.)
·2 c.c.			,,	The lateral expansions of these colonies have a tree-like appearance and are better developed than in any other concentration. Many papillae are present on the colonies, and dense colonies are present on the centre of the crowded plate. (See Pl. II, fig. 2.)
·35 c.c.			,, The expansions are however smaller and not so tree-like.	
·5 c.c.			Marginal colonies of large size show swollen edges. Big and little colonies are present in the centre of the plate.	Many papillae present on the colonies.
1·0 c.c.		The colonies on this plate show marked inhibition but are of uniform size.	All colonies small. With low magnification they show a wrinkled surface and a few lateral papillae.	As observed 5th day. (See Pl. II, fig. 3.)
1·5 c.c.	Marked inhibition.	As observed 1st day.	A few of the colonies in crowded part of plate are of a larger size than the rest.	
2·0 c.c.	Sterile.	Sterile.	Sterile.	

All observations are naked eye unless otherwise stated.

TABLE IV.

The plate in the series from which the subculture was made	Type of colony or portion of colony from which the subculture was made	Days of observation	Lactose	Glycerine	Saccharose	Broth	Indol	Motility and staining
0·1 c.c. plate	Lateral expansion of colony.	1	Af. G. 1/2	An. Gn.	An. Gn.	Cloud	+ +	+
		2	Af. G. 7/12	,,	,,			Gram
		6	Af. G. 1/2	A. 1/2 G. 1/6	,,			negative
,,	,,	1	Af. G. 1/2	An. Gn.	,,	Cloud	+ +	+
		2	Af. G. 3/5	,,	,,			Gram
		6	Af. G. 7/12	A. 1/2 G. 1/12	,,			negative
,,	Dense colony of crowded portion of the plate.	1	Af. G. 1/8	An. Gn.	,,	Cloud	+ +	+
		2	Af. G. 5/12	,,	,,			Gram
		6	Af. G. 5/12	Af. G. 1/2	,,			negative
,,	,,	1	Af. G. 1/3	An. Gn.	,,	Cloud	+ +	+
		2	Af. G. 5/12	,,	,,			Gram
		6	Af. G. 7/12	A. 1/2 G. 1/6	,,			negative
0·2 c.c. plate	Lateral expansion of colony.	1	Af. Gn.	An. Gn.	,,	Cloud	+ +	+
		2	,,	,,	,,			Gram
		6	,,	A. 1/2 G. 1/6	,,			negative
,,	,,	1	,,	An. Gn.	,,	Cloud	+ +	+
		2	,,	As. Gn.	,,			Gram
		6	,,	A. 1/2 G. 1/8	,,			negative
,,	,,	1	Af. G. 7/12	An. Gn.	,,	Cloud	+ +	+
		2	Af. G. 7/12	,,	,,			Gram
		6	Af. G. 1/12	,,	,,			negative
0·5 c.c. plate	Papillae.	1	Af. G. 5/12	,,	,,	Cloud	+ +	+
		2	Af. G. 1/2	,,	,,			Gram
		6	Af. G. 1/2	An. G. 1/6	,,			negative
,,	,,	1	Af. G. 5/12	An. Gn.	,,	Cloud	+ +	+
		2	Af. G. 1/2	,,	,,			Gram
		6	Af. G. 1/2	A. 1/2 G. 1/12	,,			negative
,,	Dense colony of crowded portion of the plate.	1	Af. G. 5/12	An. Gn.	,,	Cloud	+ +	+
		2	Af. G. 1/2	,,	,,			Gram
		6	Af. G. 5/12	A. 1/2 G. 1/10	,,			negative
,,	,,	1	Af. G. 1/2	An. Gn.	,,	Cloud	+ +	+
		2	Af. G. 7/12	,,	,,			Gram
		6	Af. G. 1/2	Af. G. 1/2	,,			negative
,,	,,	1	Af. G. 1/2	An. Gn.	,,	Cloud	+ +	+
		2	Af. G. 1/2	,,	,,			Gram
		6	Af. G. 1/2	A. 1/2 G. 5/12	,,			negative

An. = no acid. Gn. = no gas. As. = slight acid reaction.

TABLE V.

Strain	Day of observation	Arabinose	Xylose	Isodulcite	Glucose	Laevulose	Mannose	Galactose	Maltose	Lactose
(1) Lateral expansion of a colony of a 0·2 c.c. chlorhydrin plate.	1	Af. Gn.	Af. Gn.	Af. G. bub.	Af. Gn.	Af. Gn.	Af. G. bub.	Af. Gn.	Af. Gn.	Af. Gn.
	2	,,	Af. G. bub.	,,	,,	,,	,,	,,	Af. G. bub.	,,
	6	Af. G. bub.	,,	,,	,,	,,	,,	,,	Af. G. 1/4	,,
(2) Second lateral expansion of a colony of the same plate.	1	Af. Gn.	Af. Gn.	Af. Gn.	,,	,,	,,	,,	Af. Gn.	An. Gn.
	2	,,	Af. G. bub.	Af. G. bub.	,,	,,	,,	,,	,,	,,
	6	Af. G. bub.	,,	,,	,,	,,	,,	,,	,,	,,
(3) Control of normal *B. coli* (Escherich).	1	Af. G. 5/12	Af. G. 5/12	Af. Gn.	Af. G. ?	Af. G. 5/12	Af. G. 5/12	Af. G. 1/3	Af. G. 5/12	Af. G. 7/12
	2	Af. G. 1/2	Af. G. 7/12	,,	Af. G. 1/2	Af. G. 1/2	Af. G. 1/2	Af. G. 1/2	Af. G. 7/12	Af. G. 3/4

Strain	Day of observation	Cane sugar	Raffinose	Dextrin	Inulin	Salicin	Amygdalin	Glycerine	Erythrite	Adonite
(1) Lateral expansion of a colony of a 0·2 c.c. chlorhydrin plate.	1	An. Gn.	An. Gn.	As. Gn.	An. Gn.	An. Gn.	Green, no gas	An. Gn.	An. Gn.	–
	2	,,	,,	A. 1/2 Gn.	,,	,,	,,	As. Gn.	,,	–
	6	,,	,,	,,	,,	,,	,,	A. 3/4 G. 1/2	,,	–
(2) Second lateral expansion of a colony of the same plate.	1	,,	,,	As. Gn.	,,	,,	,,	An. Gn.	,,	–
	2	,,	,,	A. 1/2 Gn.	,,	,,	,,	,,	,,	–
	6	,,	,,	,,	,,	Af. Gn.	,,	Af. G. 1/4	,,	–
(3) Control of normal *B. coli* (Escherich).	1	–	–	Gp. An.	–	–	–	–	–	–
	2	–	–	Gp. A.	–	–	–	A. ? G. 1/12		–

Strain	Day of observation	Sorbite	Mannite	Dulcite	Sodium formate	Milk	Broth	Motility and staining	Indol
(1) Lateral expansion of a colony of a 0·2 c.c. chlorhydrin plate.	1	Af. Gn.	Af. G. 5/12	An. Gn.	An. Gn.	A. No clot	Cloud	+ Gram negative	
	2	Af. G. 1/3	Af. G. 5/12	Af. G. 1/8	An. G. bub.	,,	,,		
	6	Af. G. 5/12	Af. G. 5/12	Af. G. 5/12	An. G. bub.	A. Clot	,,		+
(2) Second lateral expansion of a colony of the same plate.	1	Af. Gn.	Af. G. 5/12	An. Gn.	An. Gn.	A. No clot	,,	+	
	2	Af. G. 1/3	Af. G. 5/12	Af. G. 1/4	An. G. bub.	,,	,,		
	6	Af. G. 5/12	Af. G. 5/12	Af. G. 5/12	An. G. bub.	A. Clot	,,	Gram negative	+
(3) Control of normal *B. coli* (Escherich).	1	Af. G. 5/12	Af. G. 7/12	A. 1/2 G. bub.	G. 1/2	A.	,,	+	
	2	Af. G. 7/12	Af. G. 3/4	Af. G. 7/12	G. 7/12	A. Clot	,,	Gram negative	+

B. lactis aerogenes.

We will now consider the response of *B. lactis aerogenes* to certain selecting agents.

Several experiments made with the object of selecting out non-gas-producing varieties of *B. lactis aerogenes* on monochloracetate agar proved unsuccessful and it was considered probable that the non-success was due to the very different fermentation of sugar effected by this organism as compared with *B. coli* (Escherich).

Attempts were however made with chlorhydrin agar to obtain variants of *B. lactis aerogenes* and I would like to submit here a preliminary notice of the results.

Appearance of B. lactis aerogenes *when grown at 37° C. on chlorhydrin agar plates.*

Plates containing 1 c.c. of 20 °/₀ chlorhydrin solution to 15 c.c. of agar gave fairly good growth by the second day though not so good as controls without chlorhydrin, and from the second day onward, in the crowded portions of the plates, a few colonies could be seen which were denser and more opaque than their neighbours. The difference however was slight, and not at all so marked as that shown by colonies of *B. coli* (Escherich) on plates of the same strength. After ten days' growth two colonies which projected slightly from the centre of the plate were inoculated on to agar slopes. These slopes grew slowly at 37° C.—probably because some of the chlorhydrin may have been carried over with the organisms. On the third day, however, the agar slopes were well covered with a thick growth. These cultures were then tested on a small series of carbohydrate media when one of them (Strain "6") was found to have lost the power to ferment glycerine.

The other strain retained that power in normal amount. Strain "6" was then plated out and two separate colonies tested again. They each gave the same results as before in respect of glycerine-fermentation. The loss of fermentation power was confined to glycerine, while mannite and glucose gave good yields of gas.

Strain "6" was finally tested on a large series of carbohydrate media along with a control of the normal strain and gave the results shown in Table VI.

A consideration of Table VI shows us that the chlorhydrin variety of *B. lactis aerogenes* gave acid and gas in the same sugars and alcohols

TABLE VI.

Strain	Day of observation	Arabinose	Isodulcite	Glucose	Laevulose	Mannose	Galactose	Maltose	Lactose	Cane sugar
B. lactis aerogenes	2	As. G. 1/12	Af. G. 1/10	Af. G. 7/12	Af. G. 1/2	Af. G. 5/12	Af. G. 1/4	Af. G. 1/7	Af. G. 7/16	Af. G. 5/8
Strain 6.	6	As. G. 1/12	Af. G. 1/10	Af. G. 1/2	Af. G. 1/2	Af. G. 5/12	Af. G. 1/4	Af. G. 1/7	Af. G. 1/2	Af. G. 1/2
Normal *B. lactis*	2	As. G. 1/8	Af. G. 1/8	Af. G. 2/3	Af. G. 7/10	Af. G. 1/2	Af. G. 1/2	Af. G. 4/5	Af. G. 9/10	Af. G. 2/3
aerogenes.	6	As. G. 1/8	Af. G. 1/12	Af. G. 1/2	A. 1/2 G. 1/3	Af. G. 1/3	Af. G. 1/3	Af. G. 5/8	Af. G. 1/2	Af. G. 1/2
		Raffinose	Dextrin	Inulin	Salicin	Amygdalin	Glycerine	Erythrite	Adonite	Sorbite
B. lactis aerogenes	2	Af. G. 3/4	Af. G. 1/8	–	Af. G. 1/3		–	–	Af. G. 3/4	Af. G. 1/12
Strain 6.	6	Af. G. 1/2	Af. G. 1/6	–	Af. G. 1/4		–	–	Af. G. 2/3	Af. G. 1/2
Normal *B. lactis*	2	Af. G. 5/8	Af. G. 5/8	–	Af. G. 3/8		An. G. 5/8	–	Af. G. 7/16	Af. G. 1/2
aerogenes.	6	A. 1/2 G. 1/3	Af. G. 1/8	–	Af. G. 1/4		A. 1/2 G. 1/3	–	Af. G. 5/16	Af. G. 1/2
		Mannite	Dulcite	Sodium formate	Milk	Broth	Motility		Indol	V. & P.
B. lactis aerogenes	2	Af. G. 11/12	–	–	Af.	Cloud	Non-motile		–	+
Strain 6.	6	Af. G. 4/5	–	–	A. Clot	–				
Normal *B. lactis*	2	Af. Gf.	–	–	,,	Cloud	Non-motile			+
aerogenes.	6	Af. G. 3/4	–	–	,,	–			–	

as the normal strains except in the case of glycerine. The new variety appeared to have lost a single ferment and that particular ferment was probably closely related chemically to the toxic agent used in the selection process.

The whole process was repeated and a strain (No. 13) obtained which gave the same result.

The process was again repeated with the results given in Table VII from which we see that strain "F" gave only slight fermentation of glycerine by the sixth day, and that strain "G" gave no acid or gas by the fourth day but on the sixth day showed acid without gas.

All these altered strains of *B. lactis aerogenes* reacted with Voges and Proskauer's test and were non-motile and did not liquefy gelatine.

TABLE VII.

Strain	Day of observation	Glucose	Mannite	Adonite	Glycerine
0·5 c.c. *B. lactis aerogenes* (large) A.	2	Af. G. 12/12	Af. G. 7/8	Af. G. 1/20	Af. G. 1/2
	4	A. 1/2 G. 7/12	Af. G. 9/10	Af. G. 1/10	Avs. G. 1/2
	6	A. 1/4 G. 7/12	Af. G. 4/5	Af. G. 1/9	Avs. G. 4/10
0·5 c.c. (large) B.	2	Af. G. 1/6	Af. G. 11/12	Af. G. 1/4	Af. G. 1/2
	4	A. 1/2 G. 1/2	Af. G. 7/8	Af. G. 3/10	Avs. G. 7/16
	6	A. 3/4 G. 5/12	Af. G. 4/5	Af. G. 1/4	Avs. G. 4/10
0·5 c.c. (large) C.	2	Af. G. 9/10	Af. G. 11/12	Af. G. 5/12	A. 3/4 G. 7/12
	4	Af. G. 9/16	Af. G. 9/10	Af. G. 5/12	Avs. G. 1/2
	6	A. 1/2 G. 1/2	Af. G. 5/6	Af. G. 2/5	Avs. G. 4/10
0·5 c.c. Lat. Exp. D.	2	Af. G. 12/12	Af. G. 12/12	Af. G. 1/6	Af. G. 5/12
	4	A. 3/4 G. 5/8	Af. G. 12/12	Af. G. 1/5	A. 1/2 G. 1/2
	6	A. 1/2 G. 5/8	A. 3/4 G. 11/12	Af. G. 1/6	Avs. G. 4/10
0·5 c.c. Lat. Exp. E.	2	Af. G. 7/8	Af. G. 12/12	Af. G. 1/5	A. 1/4 G. 7/12
	4	A. 1/2 G. 5/12	Af. G. 9/10	A. 1/4 G. 1/5	A. 1/4 G. 1/2
	6	A. 1/2 G. 7/12	Af. G. 5/6	Af. G. 1/5	Avs. G. 5/12
0·5 c.c. Lat. Exp. F.	2	Af. G. 3/4	Af. G. 11/12	Af. G. 5/12	
	4	A. 3/4 G. 1/2	Af. G. 11/12	Af. G. 5/12	–
	6	A. 3/4 G. 1/2	Af. G. 7/8	Af. G. 4/10	A. 1/2 G. 1/16
1·5 c.c. Big col. G.	2	Af. G. 1/4	Af. G. 4/5	Af. G. 1/3	–
	4	Af. G. 3/8	Af. G. 11/12	Af. G. 4/10	–
	6	Af. G. 1/3	Af. G. 5/6	Af. G. 3/8	Af. Gn.
1·5 c.c. Big col. H.	2	Af. G. 1/2	Af. G. 4/5	Af. G. 4/10	A. 1/2 G. 7/16
	4	A. 3/4 G. 2/3	Af. G. 12/14	Af. G. 3/8	A. 1/4 G. 1/2
	6	A. 3/4 G. 1/2	Af. G. 7/8	Af. G. 1/2	A. 1/16 G. 1/2

Avs. = very slight reaction.

After being grown on ordinary agar for two months, strains "13" and "G" were tested again on glycerine peptone water. "13" gave 1/8 of a tubeful of gas by the sixth day; the other strain gave no gas whatever after six days' growth, though it gave a late acid reaction.

I have tested on glycerine peptone water over 50 colonies of the strain of *B. lactis aerogenes* used, and have not yet met one which failed to give a good yield of gas in one day.

Theoretical considerations.

The loss of the glycerine-fermenting power of *B. lactis aerogenes* when grown in the presence of a toxic substitution product of glycerine suggests that the selection might be due to intoxication of the glycerine-fermenting bacteria by the agency of the glycerine ferment. It seems on the face of it more than a coincidence that chlorhydrin permits the selection of a non-glycerine-fermenting strain. The loss of the gas-forming power of *B. coli* when grown on monochloracetate agar was thought possibly to be due to intoxication, by the agency of the formic acid-producing enzyme, of those bacteria having a relative excess of this enzyme. This enzyme produces also acetic acid and probably therefore has a chemical affinity with acetic acid and hence with chloracetic acid. Harden has shown that the formic acid is the source of the gas, so that any toxic agent removing the formic acid-producing ferment would deprive the organism of the source of gas[1]. This explanation appears, however, doubtful, in face of the fact that chlorhydrin produces a very similar though not identical variety of *B. coli*. The intoxication and selection may be due to the CH_2Cl group common to each compound, and the acetate structure, as such, may have no specific effect.

The portion of the cell reacting with this group is obscure as there seems no reason to believe that the formic acid-producing ferment has a special affinity with it. Chloracetic acid has not been able in my experience to remove the glycerine-fermenting power of *B. lactis aerogenes*, so that we appear to have some more specific intoxication in the case of this organism with this substance.

---

[1] Harden and Walpole (1905) showed that the percentage yield of acetic acid in the decomposition of glucose by *B. lactis aerogenes* was only about one-third of that obtained when *B. coli* (Escherich) fermented this sugar. Now if the acetic acid producing enzyme were the agent of intoxication one would expect chloracetate to be much less effective in the case of *B. lactis aerogenes* than in that of *B. coli* (Escherich). This appears to be the case.

The possibility of ferment intoxication led me to try lactose phenylhydrazone as a selecting agent hoping therewith to obtain a non-lactose-fermenting variant. It was soon evident that the intoxication of the bacteria was conditioned by the NH group in this substance. The hydrogen was therefore replaced by a methyl group when the toxicity of the substance was found to be greatly reduced. This methyl derivative is being used at present but the work is not sufficiently advanced to permit of any statement of its effects as a selecting agent.

Ehrlich has held for some time that the nutriceptors play a part in cell intoxication. To prove this, one ought to be able to show that the surviving cells, after a selective intoxication with the given agent, are not able to use a definite food which the original cells used, or at least do not use it to the same extent. This view does not appear as yet to rest on any experimental basis[1].

SUMMARY.

1. *B. coli* (Escherich) produces papillated colonies and shows marked variability in the size of its colonies when it is grown on agar to which phenylacetic acid has been added in the form of the sodium salt.

The big and little colonies produce about the same amount of gas when tested on glucose.

2. When *B. coli* (Escherich) is grown on agar containing monochlorhydrin it throws off variants similar to those produced when grown on monochloracetate of soda media. Speaking broadly they ferment alcohols with gas formation, and sugars without gas formation.

3. *B. lactis aerogenes* grown on monochlorhydrin agar gives rise to variants unable to ferment glycerine.

4. In cases of inhibitory bacterial selection by chemical agents a careful comparison of the surviving cells with the original strain from which they were derived is calculated to indicate that portion or function of the cell which is implicated in the cell's intoxication. This question does not seem to have been attacked hitherto from this standpoint.

5. The cell ferments by virtue of their specific chemical affinities may play a part in cell intoxication.

---

[1] It appears probable that the cell enzymes frequently play a secondary part in cell intoxication. We know for example that carbolic acid is rendered much more germicidal by the addition of acids. Now in many media the cell enzymes will produce acids, hence it is probable that carbolic acid selections of bacteria commonly result in the development of new strains with impaired fermenting power.

I desire to express my indebtedness to the authorities of the Lister Institute and to the "Constance Research Fund" administered by Dr E. C. Hort for all the experimental facilities which made the work of this paper possible.

My thanks are likewise due to Dr Ledingham for valuable help and criticism during the course of this research.

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Note: For description of Plate II, see Table III, p. 40.

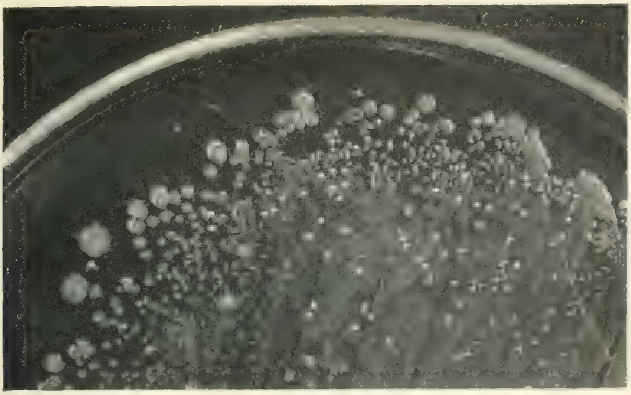

Fig. 1. For description see Table III.

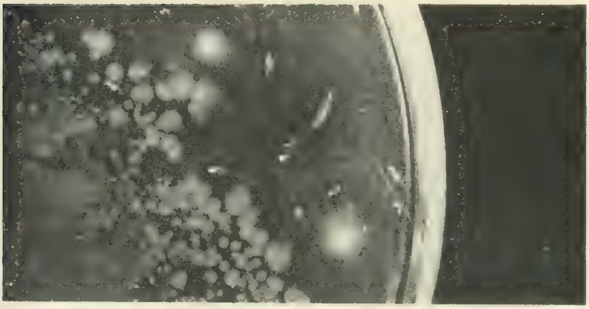

Fig. 2. For description see Table III.

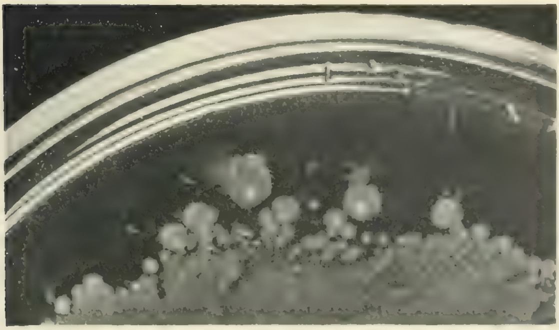

Fig. 3. For description see Table III.

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THE RELATION OF CONCENTRATION OF FOOD SUPPLY TO THE GENERATION-TIME OF BACTERIA.

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(From the Bacteriological and Biochemical Departments, The Lister Institute, London.)

(With 3 Charts.)

MANY investigations have been undertaken to determine the generation-time of bacteria but although the effects of temperature and other factors have been considered, little attention has apparently been paid to the influence of the concentration of the culture medium employed.

In most of the papers on this subject the concentration of peptone used is not mentioned at all and hence it is difficult to compare the results obtained.

The influence of glucose and of salts has also been little investigated, the experiments on this point being designed only to determine between what limits of concentration there was any growth at all.

The question is of some interest inasmuch as exhaustion of food supply is probably a factor in bacterial selection. Thus Beyerinck considers selection to be a feature of slow growth, and this receives support from the behaviour of *B. typhosus*, in a dulcite medium, for, while growth is still proceeding rapidly according to a logarithmic law, there is but little evidence of selection, which only takes place later when the growth has become comparatively slow (Penfold, 1911).

Generation-time of B. typhosus *in Peptone-water.*

The following experiments were carried out to see to what extent the generation-time of *B. typhosus* at 37° C. was influenced by the concentration of peptone used. This varied from 0·0125 % to 1·25 %, the amount of NaCl present being kept constant at 0·5 %. Tubes containing 5 c.c. of these various media were then inoculated with a standard drop of a suitable dilution of a 24 hour broth culture of *B. typhosus*. It was found that with such a culture diluted to 1 in 5000, the standard drop employed gave an initial bacterial count of from 200–500 organisms per c.c. of the inoculated broth examined immediately. The cultures were then incubated at 37° C. and at known intervals of time definite volumes were plated out and the number of bacteria per c.c. calculated. It was arranged that each plate should contain about 200 colonies and, where dilution was necessary, this was carried out in 0·8 % saline. The first observations of which account is taken in the curves shown below were not made until sufficient time had elapsed to ensure the period of lag being over.

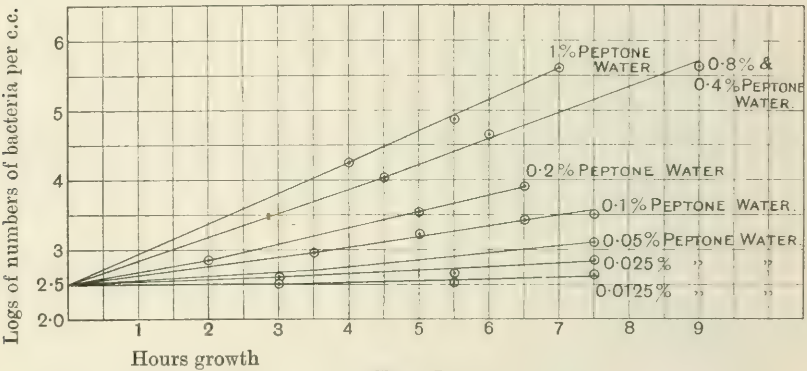

Chart I.

In Chart I the logarithms of the number of bacteria in unit volume have been plotted against the time of growth, and it will be seen that there is a rapid decrease in the rate of growth as the concentration of peptone falls below 1 %, until with 0·01 % of peptone little multiplication takes place. It will also be noticed that 0·8 % and 0·4 % peptone both gave identical results although lower than the 1 %. In a second sample of peptone, all concentrations above 0·4 % gave practically the same generation-time. From the above results the generation-times were calculated for each concentration of peptone from the formulae

$2^n = \frac{b}{a}$ where a = no. of bacteria at beginning
b = ,, ,, ,, end,
n = no. of generations,
and $G = \frac{T}{n}$ where G = generation-time and
T = time of experiment.

Only that period in which growth was proceeding according to the logarithmic law was taken into account in these calculations.

In Chart II the generation-times calculated from the above experiments have been plotted against the concentrations of peptone, the two curves representing two different samples of peptone.

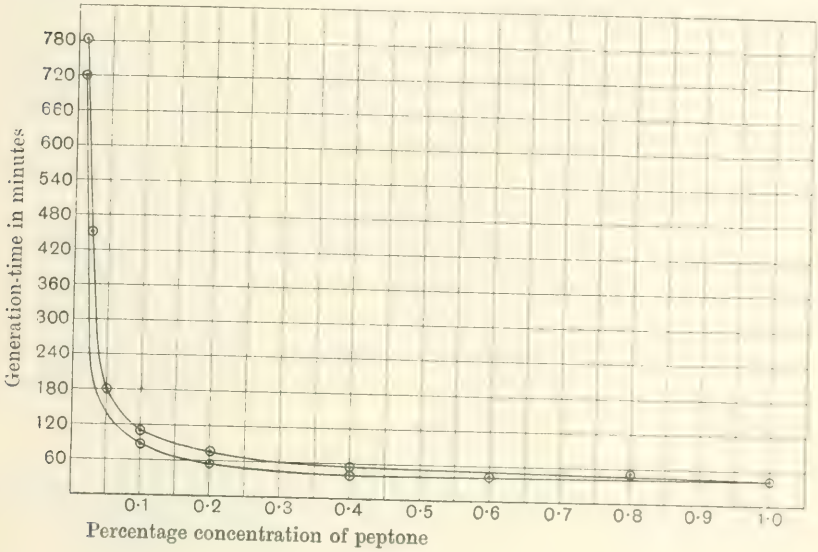

Chart II.

The generation-time increases but slowly until the concentration of peptone falls below 0·4 %. After this however it becomes rapidly greater and with 0·012 % is almost infinite.

The following table gives the generation-times for each sample of peptone examined.

TABLE I.

Percentage concentration of peptone. (C.)	Generation-time in minutes. (T.)			
	Sample I.	C · T	Sample II.	C · T
1·25	—	—	42	—
1·0	39	—	—	—
0·8	49	—	—	—
0·6	—	—	42	—
0·4	49	—	40	—
0·2	77	—	51	10·2
0·1	111	11·1	84	8·4
0·05	181	9·05	—	—
0·025	450	11·2	—	—
0·0125	783	9·8	723	9·04

It will be noticed that with concentrations below 0·2 % in one case and 0·4 % in the other the product of concentration of peptone and generation-time is a constant within the limits of the experimental error, this being about 10 %. It will be seen that there is a considerable variation in the generation-times when different samples of peptone are used. In each case however the shortest time obtained was the same namely from 39–40 minutes.

Hehewerth's (1901) result was 45 minutes at 37° C. and Lane-Claypon (1909) found 33 minutes at 34·3° C. so that our value lies between the two.

Effect of addition of Glucose to the Peptone.

We next tried the effect of adding small amounts of glucose to the peptone tubes. It was to be expected that with low peptone concentrations the generation-time would be reduced by this procedure and this proved to be the case. The amount of glucose added was 0·175 %.

The experiments are at present incomplete but the results so far obtained are shown in Chart III.

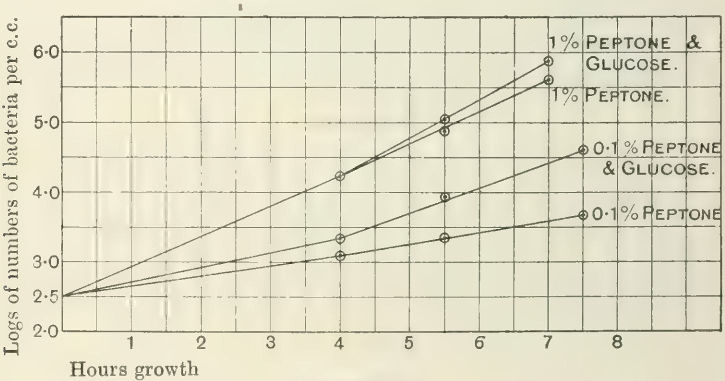

Chart III.

In this chart is shown the effect of adding 0·175 % of glucose to tubes containing 1·0 % and 0·1 % peptone respectively. In the case of the 1·0 % peptone the rate of growth is not much increased. Only the later part of the curve is affected, the generation-time being decreased from 39 minutes to 34 minutes. In the other case, however, the rate of growth is greatly increased by the small amount of glucose added and the generation-time decreased from 111 minutes to 50 minutes or about 50 %. In further experiments it is proposed to determine the effect on the generation-time of concentrations of peptone higher than 1 % and of larger additions of glucose.

We desire to express our indebtedness to Dr J. C. G. Ledingham for help in this research.

SUMMARY.

1. The generation-time of *B. typhosus* in 1 % peptone at 37° C. is 40 minutes.

2. The rate of growth of *B. typhosus* is greatly influenced by the concentration of peptone in nutrient medium when the peptone is below 0·4 %.

3. *Below* 0·2 % *the generation-time is inversely proportional to the concentration of peptone used.*

4. The addition of 0·175 % of glucose to a medium containing only 0·1 % of peptone lowers the generation-time by about 50 %.

With 1·0 % peptone this effect is less marked.

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LVII. ATTEMPT TO SEPARATE THE ANTIGEN FROM THE NUCLEOPROTEIN OF THE PLAGUE BACILLUS BY FILTRATION THROUGH GELATIN.

By SYDNEY ROWLAND, M.A., M.R.C.S.

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The antigen of plague as we have established in the course of these reports is intimately associated with a nucleoprotein soluble in dilute salines. By extracting this nucleoprotein from the bacillus the contained antigen (for rats) can be obtained. The relations that exist between the amount of antigen contained in the bacillus and the amount of antigen associated with the nucleoprotein extractable from the bacillus show that in the process of extraction there is little or no loss. The process is, within the limits of experimental error imposed on all measurements based on animal experiments, a quantitative one. In this respect it differs from the process of Lustig and Galeotti[1]. These observers used a 0·75 % solution of NaOH as an extraction medium and the quantity of nucleoprotein obtained by their method necessary to immunise a rat is given by them as 0·36 mg. If this amount be compared with the amount of nucleoprotein associated with one protecting dose of the extract obtained by the sulphate of soda method, a striking contrast is apparent. A dose of extract containing 0·0001 mg. protects about 60 per cent. of the rats which receive it (*Journal of Hygiene*, vol. x. (1910), p. 559).

Not only is the associated quantity of nucleoprotein much smaller when the sulphate process is employed, but as far as can be seen there is no loss of antigen in the process. Thus the relations by weight of the nucleoprotein to the bacillus (both weighed dry) are as about ten to one, *i.e.*, from one gram of dry bacilli about 0·1 gram of nucleoprotein is obtained. The protective values of the bacillus and the nucleoprotein obtained from it are about the same. Thus the mortality after the injection of rats with 0·03 mg. of whole bacillus is about 25 %, while the mortality after injecting 0·001 mg. nucleoprotein is about 12 %, the mortalities in both cases occurring after the injection into the test animals of the same quantity of the same virulent broth culture. It is very difficult to obtain exact experimental proof that no loss of antigen occurs on extraction; the figures just quoted show that there is no appreciable loss. In 0·03 mg. of whole bacillus there is 0·003 mg. of nucleoprotein. Inoculation of one-third of this amount was followed by a better immunity than followed the inoculation of 0·03 mg. of whole bacillus.

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[1] *Deutsch. med. Woch.* 1897, pp. 25, 227, 289.

The question of the relations that exist between the nucleoprotein and the antigen might be elucidated by a study of the effects of filtering the nucleoprotein solution through gelatin, as the gelatin filter serves as a ready means of removing proteins from their solutions. Thus it would be possible to ascertain whether the antigen was so closely bound to the nucleoprotein that gelatin might or might not effect a separation more or less complete between them. More especially was this thought to be worth investigating in view of the results obtained by hydrolysing the nucleoprotein.

Hydrolysis was found to affect the nucleoprotein whilst leaving the antigen intact (vol. XI. (1911), supplement, p. 35). It was possible, therefore, that one of the effects of hydrolysis was to effect a separation between the antigen and the nucleoprotein. The data required are (1) the nucleoprotein content and antigenic value of a solution: (2) the same figures with regard to the same solution after passing through gelatin. With a view to obtaining information on this question the following experiments were carried out.

A solution was prepared. Its antigenic value is given by the experiments in the following table:

Dose	No. rats	No. survived	% survived
0·01 mg.	14	12	86

Thus 0·01 mg. protected 86 % of the rats inoculated with it.

The solution was then allowed to autolyse for a month. It contained originally 2 mg. nucleoprotein per cubic centimetre. It was then filtered through 10% gelatin under a pressure of 40 atmospheres. The particular method employed was that devised by Martin[1].

Under this pressure filtration was rapid and it was feared that the gelatin was not effecting a complete separation between the proteins

[1] *Journal of Physiology*, vol. XX. (1896) p. 364.

and the solution. The solution was accordingly passed through the filter several times until a small portion of the filtrate gave no reaction on boiling after acidification with acetic acid. The apparent passage of the nucleoprotein through the gelatin might be used as an argument that the constituent molecular aggregates are small. It must be remembered that the solution was, considered as a proteid solution, very dilute. It contained originally only 2 mg. per c.c. and after the month's lysis it contained less. Thus the solution was only 0·2 %. The statements that are given as to the impermeability of 10 % gelatin to protein solutions are all based on the examination of much stronger solutions than this. It is possible that very dilute solutions of all proteins pass through gelatin. Indeed, the fact that continued passage of the dilute solution used in this experiment did result in a more nearly complete arrest of the protein points to this conclusion.

After the filtration was complete a further test was made for the presence of protein. It was found that in 100 c.c. an amount of protein approximating to 1 mg. was present.

It now remained to determine the vaccinating value of this solution. The experiments performed to ascertain this point are given in the following table:

Dose	No. rats	No. survived	% survived
1 c.c.	19	16	84

The quantity of nucleoprotein contained in the vaccinating dose was 0·01 mg.

The amount of protection—84 %—is practically identical with the protection obtained by the use of the solution before hydrolysis.

Now the hydrolysis has increased the ratio of antigen to nucleoprotein, for it has diminished the amount of protein recoverable by boiling in the presence of acetic acid (*Journal of Hygiene*, vol. XI. (1911), supplement, p. 27), and it has not diminished the amount of antigen (*loc. cit.*). We should expect, therefore, that even if the gelatin has not diminished the ratio of protein to antigen, the filtrate would show a high immunising value. The experiment quoted does not determine the ratio of antigen to protein. The protection afforded is high—over 80 %. It was possible that a smaller quantity of the filtrate would give the same amount of protection. If a smaller amount of the filtrate was found to give a smaller protection then we must conclude that the ratio of antigen to nucleoprotein was not materially altered by the filtration. This, as the following experiments show, was found to be the case:

Dose	No. rats	No. survived	°/₀ survived
1/20 c.c.	23	13	56
1/200 c.c.	23	13	56

Reference to the previous reports (vol. X. (1910), p. 559 and vol. XI. (1911), supplement, p. 40) will show the amount of protection following the inoculation of a one-month's hydrolysed extract. Thus on p. 40 of the second report 53 °/₀ survived after a vaccinating dose of 0·001 mg., an amount comparable with that present in the solution used above.

We must conclude that filtration through gelatin has effected no separation of antigen from protein.

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LVIII. BESREDKA'S METHOD OF VACCINATION.

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A METHOD of vaccination that has achieved considerable reputation is that advocated by Besredka of the Pasteur Institute, Paris[1]. "Vaccination par les virus sensibilisés," as he terms his method, consists essentially in the use of killed cultures of the organism concerned. In this it does not differ, so far as concerns plague, from the method of Haffkine. But Besredka claims that, if the killed organisms are submitted to a preliminary soaking in a serum containing the specific antibodies to the organism concerned, these antibodies neutralise the toxic substances contained in the bacilli, which can then be used as a vaccine having the advantage of being atoxic.

The present position of the author of this method will be found described in the *Bulletin de l'Institut Pasteur* (1910), vol. VIII. p. 241, where he says "le vaccin antipesteux sensibilisé est depourvu de toute action toxique."

Now in the case of the plague bacillus we can extract the specific endotoxine (for rats), consequently we are in the position of being able to control this claim. Assuming that a given quantity of plague bacilli will normally yield a certain definite amount of endotoxine, then it is obvious that, if Besredka's explanation of the principles underlying his method be correct, the same quantity of plague bacilli will, after soaking in the antiserum, yield a smaller amount of endotoxine or none at all.

The following experiments were undertaken to determine

(1) The toxicity of a "whole" vaccine prepared according to Besredka's method.

(2) The amount of endotoxine that could be extracted from plague bacilli before and after soaking (sensitising) in antiserum.

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[1] See the reviews by Besredka in *Bulletin de l'Institut Pasteur*, vol. VIII. (1910), p. 241 and vol. X. (1912), p. 529.

Experiment I. Determination of the toxicity of a vaccine prepared according to Besredka's method.

In this experiment the relative toxicity of three vaccines is determined. The three vaccines are comparable in every way except that in the case of vaccine 1, the bacilli were soaked (sensitised) in an antiplague serum; in the case of vaccine 2 they were soaked in normal horse serum; and in the case of vaccine 3 they were soaked in physiological salt solution.

The serum employed in the case of vaccine 1 was prepared by immunising a horse by means of the endotoxine of the plague bacillus as already described (*Journal of Hygiene*, vol. XI. (1911), supplement, p. 11). At the time of the experiment this serum was of such a strength that one cubic centimetre neutralised 150 lethal doses of endotoxine. The proportion of serum to bacilli was so arranged that there was present in the quantity of serum employed sufficient antitoxin to neutralise four times the amount of endotoxine present in the bacilli.

These relations having been determined by preliminary experiments, 100 Roux bottles of agar were inoculated with plague and incubated for four days at 34° C. The bottles were then heated to 60° C. for an hour to kill the bacilli. This is the temperature and duration of heating employed by Besredka. The growth was washed off in salt solution (0·8 %) and the bacilli twice washed in salt solution in the centrifuge. After the last washing the bacilli were again centrifuged and 41 grammes of paste obtained. This paste contained 13·84 % of solids when dried at 105° C. Ten grammes of the paste were put in each of three flasks containing glass beads and there were added 35 c.c. of antiplague serum, normal serum and salt solution respectively. When a uniform distribution of the organisms throughout the respective fluids had been effected the flasks were put aside in a cool place till the following day. Meanwhile the quantity of bacilli in emulsion 3 was determined. It was found that the strength of this emulsion as determined directly corresponded with the strength as calculated from the analysis of the paste from which it was made. We may take it then that the paste of organisms was homogeneous, that the process of emulsification was adequate and that the three emulsions were comparable in the amount of organisms they contained.

The lethal dose of the three emulsions was then determined by subcutaneous inoculation into rats. Two rats were used for each dose:

Dose mg.	Emulsion 1	Emulsion 2	Emulsion 3
10	Died 2 days	Died 2 days	Died 2 days
	,,	,,	Survived
9	,,	Survived	Died 2 days
	,,	,,	Survived
8	,,	,,	Died 2 days
	Survived	,,	,,
7	Died 3 days	,,	,,
	Survived	,,	Survived
6	,,	,,	Died 2 days
	,,	,,	,,
5	,,	,,	Survived
	,,	,,	,,
4	,,	,,	,,
	,,	,,	,,
3	,,	,,	,,
	,,	,,	,,
2	,,	,,	,,
	,,	,,	,,
1	,,	,,	,,
	,,	,,	,,

The lethal dose of the three emulsions does not differ to any great extent. Outside the error of this kind of experiment there is no difference discoverable between the lethal dose of the organism that had been soaked (sensitised) in the antiplague serum and those that had been treated simply with salt solution. A slight apparent difference is noticed in favour of the organisms that had been sensitised in the normal horse serum.

A repetition of this experiment showed no diminution of toxicity in the case of the bacilli treated with serum.

Experiment II. Estimation of the amount and toxicity of the endotoxine that can be extracted from plague bacilli before and after soaking in antiplague serum, normal horse serum and salt solution.

A paste of organisms was prepared as in the last experiment: 32·5 grammes of paste were obtained containing 15·6 % solids.

Three suspensions of the paste were made as in the last experiment.

Suspension 1 contained 10 gr. paste in 37·5 c.c. antitoxic serum.
,, 2 ,, ,, ,, normal horse serum.
,, 3 ,, ,, ,, salt solution.

The three suspensions were left in a cool place until the following morning. They were then centrifugalised and the paste of organisms washed free from serum proteins. The final washed paste obtained was mixed with twice its weight (in each case) of anhydrous sulphate of soda. The semi-fluid mass thus formed soon set to a solid mass which was reduced to powder. The full description of this process of extracting the endotoxine of the plague bacillus has already been given in these reports (vol. x. (1910), p. 553). The powder was dissolved in warm distilled water and in such a volume as to form a saturated solution of sodium sulphate. After filtering off the solution the residue on the filter consisting of plague bacilli, sensitised or not as the case might be, was extracted with distilled water. The same amount of water was of course used in each case. Three extracts were thus obtained which contained the endotoxine from the three portions of bacilli which had been treated in the three ways indicated. Each extract was filtered through hardened filter paper to remove the bodies of the bacilli and the three filtrates examined as follows.

First the amount of nucleoprotein in each was determined by boiling a portion after acidification with acetic acid, drying and weighing the precipitate obtained. The weights of nucleoprotein in the three extracts in milligrammes per c.c. was as follows:

No. 1	No. 2	No. 3
6·62	6·34	6·66

These figures are very similar. There is no appreciable difference in the weight of endotoxine (nucleoprotein) extracted in the three cases.

Having thus failed to find any difference in the quantitative yield of nucleoprotein in the three cases, it remained to ascertain whether there was any qualitative difference in the three extracts. For this purpose the lethal doses of the three extracts were determined on rats. For the purposes of this determination the three extracts were made up to such a strength that 5 c.c. of each extract contained 1 milligramme of nucleoprotein. The three solutions were inoculated subcutaneously into rats as follows, two rats being used for each dose:

Dose mg.	Extract 1	Extract 2	Extract 3
1·0	Died 1 day	Died 1 day	Died 1 day
	,,	,,	,,
0·8	,,	,,	,,
	,,	,,	,,
0·6	,,	,,	,,
	,,	,,	,,
0·4	Died 2 days	,,	,,
	Survived	Died 2 days	,,
0·2	Died 1 day	Died 1 day	,,
	Died 2 days	Survived	Died 3 days
0·1	Survived	Died 1 day	,,
	,,	Survived	Survived

The minimal lethal dose of the nucleoprotein obtained from bacilli that had been sensitised in immune horse serum was thus substantially the same as the minimal lethal dose of the nucleoprotein obtained from the bacilli that had been treated (sensitised) in normal horse serum or in saline. The yield of nucleoprotein was also the same in the three cases.

From this experiment, which was repeated with similar results, it is concluded that on sensitising the plague bacillus in an antitoxic serum no permanent neutralisation of the endotoxine takes place.

The control experiment in which the bacilli were treated in salt solution shows that the effect of sensitising in either anti or normal serum is nil as regards the amount of nucleoprotein or the toxicity of the nucleoprotein.

I am therefore unable to confirm Besredka's statement that sensitised organisms yield an atoxic vaccine, for the whole organisms after sensitisation are just as toxic as before and no neutralisation of the endotoxine takes place as the result of the sensitising.

As the immune serum neutralised the endotoxin after extraction from the bacilli it must be concluded from these experiments that the antitoxine cannot enter the bodies of bacilli killed by heat.

There is, however, evidence that Besredka's vaccine is possessed of good immunising power and users of it have reported favourably as to the minimum of discomfort following its inoculation. A great point of this latter property is made by Besredka. Paladino-Blandini (*Annali d'Igiene sperimentale* (1905), pp. 295, 411), after an exhaustive examination of several methods of antityphoid vaccination, speaks in terms of high praise as to this property of Besredka's vaccine. Dopter (*Annales de l'Institut Pasteur* (1909), vol. XXIII. p. 677; *C. R. Soc. Biol.* vol. LXIV. (1907), p. 379), working with dysentery, speaks to the same purpose.

Besredka attributes the advantages of his sensitised vaccines firstly to an actual neutralisation of the endotoxin and secondly to sensitisation (opsonisation) of the bacteria. For the former I can find no support from my experiments with plague, but immune horse serum does contain opsonin. At one time Besredka recommended the use of normal horse serum in the preparation of his sensitised vaccine as being equally efficacious. Normal serum, however, can hardly be supposed to be antiendotoxic or possessed of any considerable specific opsonising power.

A further possibility is that after soaking in immune serum the bacilli are more readily lysed when placed under the skin. In this respect treatment with normal horse serum may be advantageous, for whilst engaged in some investigations into the mechanism of plague immunity I found that in the normal horse a natural amboceptor for the plague bacillus exists in considerable quantity. It is possible that in Besredka's method this natural amboceptor is responsible for sensitising the bacilli so that they dissolve more rapidly after injection. Whilst at first Besredka employed specific sera for his sensitisation, later he abandoned these for the use of normal horse serum. Later still he reverted to the use of specific sera and makes the statement that it is necessary for these to be highly agglutinating.

The main experiments that led to the recognition of the normal amboceptor in the serum of normal horses are as follows:

(1) 80,000,000 virulent living bacilli were added to 1 c.c. of fresh normal horse serum. The serum was kept at 37° C. No multiplication took place and at the end of 24 hours the serum was sterile.

(2) 83,000,000 living virulent bacilli were added to 1 c.c. of the same serum as was used in the last experiment. The serum had been heated to 58° C. for one hour. Multiplication took place and followed the same curve when the numbers were plotted against time as the same number of bacilli inoculated into broth.

(3) 10,000,000 living virulent bacilli were added to one c.c. of the same serum that had been heated. At the same time 0·04 c.c. of fresh normal rat serum was added to the tube. No multiplication took place and at the end of four hours only half a million bacilli were recognised.

The growth and fate of living plague bacilli were traced in horse serum by the use of a method of direct microscopical observation. Specially constructed observation slides were used which, combined with a very perfect method of dark ground illumination, allowed of a record of the appearances of the living organisms and of their number being made as time advanced (see below, p. 362).

The further fact that so far as they have been examined all antiplague sera prepared by Yersin's or some equivalent method are strikingly deficient in specific plague antitoxine supports the explanation above given.

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LIX. THE RELATION OF PSEUDO-TUBERCLE TO PLAGUE AS EVIDENCED BY VACCINATION EXPERIMENTS.

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THE resemblance of the bacillus of pseudo-tubercle of rodents to that of plague is very striking. The work of Galli Valerio[1], Zlatogoroff[2], and MacConkey[3] bears evidence of this. It has even been suggested that the pseudo-tubercle bacillus should be rechristened and be known as the pseudo-plague bacillus. There is no constant morphological or staining character which will enable us to distinguish the one from the other, nor do we receive much assistance towards the attainment of this end from a study of the cultural and biological properties of the two organisms[4]. The differences are merely differences of degree. It is the same with regard to the agglutination, precipitin and, as far as they are known, complement deviation reactions (J. Henderson Smith[5]). The post-mortem appearances in both diseases may be so strikingly similar that one can do no more than hazard a guess as to which bacillus is the cause of them. The similarity in the appearances of a guinea-pig that has died of pseudo-tubercle to those of a guinea-pig that has died of plague is so great as often to amount to identity. The only well-defined difference between the two organisms is one of virulence, for whereas the rat is hardly, if at all, susceptible to pseudo-tubercle it is very susceptible to plague. The guinea-pig is usually regarded as being equally susceptible to either.

These great resemblances led Zlatogoroff and MacConkey to test the question of the possibility of immunising rats and guinea-pigs against plague by means of the pseudo-tubercle bacillus. Zlatogoroff failed to obtain cross-immunisation but MacConkey concluded that it was possible to immunise both rats and guinea-pigs against plague by means of inoculation of cultures of the pseudo-tubercle bacillus. In the case of rats he employed a living culture as his vaccine, whilst he found that with guinea-pigs either a living or a filtered autolysed extract was efficaceous. His conclusions being drawn from only a relatively small number of animals it was considered of importance that they should be confirmed or refuted, and if found to be correct that the scope of the enquiry should be extended so as to obtain amongst other things some estimate of the extent of the immunity produced.

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[1] *Cent. f. Bakt. Orig.* vol. xxxiii. (1903), p. 321. [2] *Ibid.* vol. xxxvii. (1904), p. 513.

[3] *Journal of Hygiene*, vol. viii. (1908), p. 335.

[4] MacConkey, *Journal of Hygiene*, vol. v. (1905), p. 350; see also *Ibid.* vol. viii. (1908), p. 305.

[5] Unpublished observations.

The immunity to plague conferred on guinea-pigs by pseudo-tubercle.

A vaccine was prepared from a culture of pseudo-tubercle that came originally from Professor Pfeiffer.

Preparation of the vaccine. Twelve Roux flasks were inoculated with this culture and incubated for three days at 35° C. Five c.c. of chloroform were then introduced into each flask and the flasks were again incubated for four hours. At the end of this time 10 c.c. of salt solution were introduced into each flask and the growth emulsified. To 26 c.c. of this emulsion were added 134 c.c. of salt solution. This constituted the vaccine. Each guinea-pig received 0·5 c.c. of this vaccine subcutaneously and after an interval of 15 days a second dose of 1·0 c.c. It was reckoned that the first dose corresponded to $\frac{1}{8}$th agar tube and that the second dose corresponded to $\frac{1}{4}$th agar tube of average growth.

A month after the second vaccination each pig received $\frac{1}{10}$ c.c. of a broth culture of living virulent plague subcutaneously. At the same time 10 control pigs received a similar dose.

There were 44 guinea-pigs vaccinated and not a single one died of plague or was noticed to be even ill, whereas nine out of the ten control pigs died of plague. The vaccination has, therefore, given rise to complete protection.

The immunity to plague conferred on rats by pseudo-tubercle.

MacConkey obtained indications of some degree of resistance to plague in rats which had previously been inoculated with living pseudo-tubercle bacilli. Of thirteen rats which were so treated five died of plague when tested five months after the last immunising injection. Of his five controls all died.

Accordingly an experiment was made on rats using the same vaccine and the same method of immunisation as in the case of guinea-pigs. Thirty-seven rats were used for the experiment, but no evidence was obtained that any protective effect was produced when tested with living virulent plague four weeks after the second dose of vaccine.

The difference in the reaction of the two classes of animals to the vaccination appears all the more remarkable when we remember the facility with which rats can be immunised against plague by previous treatment with dead plague cultures and the difficulty with which by this means guinea-pigs are rendered immune to the same disease.

The immunity to plague conferred by the nucleoprotein of the pseudo-tubercle bacillus on rats and guinea-pigs.

As in the case of the plague bacillus a water-soluble nucleoprotein can be extracted from the bacillus of pseudo-tubercle. The same methods which have already been described in these reports (this *Journal*, vol. x. (1910), p. 553) were applied to the pseudo-tubercle bacillus and an extract prepared similar in its chemical characteristics to that obtained from the plague bacillus. Thus, to give an example of one preparation, 19 Roux bottles were inoculated and after four days' incubation at 34° C. a good growth was obtained. This was swept off in salt solution and the bacilli centrifuged down. The paste of organisms was emulsified in salt solution and again centrifuged in order to wash the bacilli. This process was repeated again. No chloroform was used in the process. To the final washed paste an equal weight of anhydrous sulphate of soda was added. The semi-liquid mass thus produced was rubbed in a mortar until on cooling it became a dry powder. The powder was dissolved in twice its weight of warm distilled water. A solution of sulphate of soda is obtained by this means which is of such a strength as to completely precipitate all the bacterial proteins. This solution was filtered off and the residue of organisms suspended in distilled water to extract. After an hour the extract was filtered through hardened paper and a clear fluid obtained. This clear fluid was found to contain 10·8 mg. of nucleoprotein per c.c. Unlike the extract obtained from the plague bacillus by the same treatment it was without toxicity for either rats or guinea-pigs. Guinea-pigs and rats survived doses of 10 mg.

Numerous experiments were made to determine whether this extract was possessed of any immunising value against living plague but all with a negative result. Thus, in one series of 26 rats the mortality of the vaccinated animals was 18 % greater than the mortality of the controls and in another series of 26 guinea-pigs it was only 6 % less. In both these series the vaccinating dose of the nucleoprotein was 0·01 mg. and the test dose of plague was given two weeks after the vaccination.

Further experiments were made under the same conditions of testing as have been used in determining the immunity against plague following the inoculation of various vaccines, the technique of which is detailed in the first of these reports. The results may be tabulated as follows:

	Dose	No. animals	Result	% mortality
Rats	0·1 mg.	23	12 died of plague	52
	0·01 ,,	5	5 ,, ,,	100
Guinea-pigs	0·1 ,,	22	13 ,, ,,	59
	0·01 ,,	28	19 ,, ,,	68
Controls not vaccinated:				
Rats		19	14 ,, ,,	73
Guinea-pigs		15	9 ,, ,,	60

In the case of rats which received the 0·1 mg. dose a slight immunity is noticed.

In the case of the guinea-pigs no immunity is evident.

We must conclude that by the method employed the substance contained in the pseudo-tubercle bacillus which immunises guinea-pigs against plague cannot be extracted.

If it cannot be extracted and if it is not destroyed by the process used in attempting to extract it, it must be insoluble in dilute saline and remain in the bodies of the bacilli after the extraction. No experimental determination of this question has yet been made, but assuming that it remains in the bodies of the bacilli it presents a striking contrast to the antigen of plague which is effective in the case of rats.

The plague antigen for rats is easily extracted from the plague bacillus, which bacillus under similar treatment yields little or no antigen for guinea-pigs. A plague antigen for guinea-pigs is contained in the pseudo-tubercle bacillus from which by the method employed it cannot be extracted. The antigens for the two animals are, therefore, different.

The mechanism of immunity in the two classes of animals is therefore different—a fact of great importance in the art of vaccination. And it therefore behoves us to be careful in applying the results obtained from the study of a particular species of animal to man.

Note on the toxicity of the plague nucleoprotein for guinea-pigs. The nucleoprotein obtained from the plague bacillus and which, as has been shown, is invariably toxic and possessed of antigenic powers for rats is almost without action on guinea-pigs.

This conclusion is the result of repeated trials. The following test of a sample that was prepared without chloroform in order to eliminate the possible neutralising action of this substance may be given:

Dose	Rats	Result	Dose	Guinea-pigs	Result
5·4 mg.	1	Died 3 hours	27 mg.	1	Survived
	2	Died 1 day		2	,,
2·7 ,,	3	,,	18 ,,	3	,,
	4	,,		4	,,
1·3 ,,	5	,,	2·7 ,,	5	,,
	6	,,		6	,,
0·1 ,,	7	,,	1·3 ,,	7	,,
	8	,,		8	,,

Coincident with this absence of toxicity, there is a minimum amount of protective power as the following table shows:

No. guinea-pigs	Protecting dose	Result	% survived
20	11 mg.	14 died	30
20	5 ,,	17 died	15
Controls received living plague only:			
40	—	37 died	7

The slight balance in favour of the vaccinated animals is not significant in view of the fact that pseudo-tubercle is a common disease amongst laboratory guinea-pigs and the knowledge we now have as to the protective value of this bacillus against plague.

Summary.

It will be as well to summarise the above facts:

1. *The plague bacillus is equally virulent for guinea-pigs and rats, but whereas it is easy by either the whole bacillus or the contained nucleoprotein soluble in saline to immunise the rat against plague it is almost impossible to immunise the guinea-pig, either by means of the bacillus or its nucleoprotein.*

2. *The pseudo-tubercle bacillus is almost without pathogenicity for rats whereas the guinea-pig is susceptible to it.*

3. *It is easy to immunise the guinea-pig against plague by means of the pseudo-tubercle bacillus; very difficult to immunise the rat.*

4. *It was not found possible to immunise either the rat or the guinea-pig against plague by means of the nucleoprotein contained in the pseudo-tubercle bacillus.*

5. *Whereas the nucleoprotein of the plague bacillus grown on broth agar is toxic for rats it is hardly so at all for guinea-pigs. The nucleoprotein obtained from the pseudo-tubercle bacillus is relatively innocuous to either rats or guinea-pigs.*

These facts suggest that for a bacillus or bacillary product to act as an efficient vaccine it must in its original condition be possessed of toxic action for the animal to be protected. Thus the plague bacillus is toxic for rats and can be used as a vaccine, the nucleoprotein obtained from it is toxic for rats and will protect them, but it is not toxic for guinea-pigs and will not protect them. The pseudo-tubercle bacillus is pathogenic for guinea-pigs and will protect them, the nucleoprotein obtained from it is toxic to neither rats nor guinea-pigs and is incapable of protecting either.

This statement appears at first sight as opposed to the facts established in a previous report as to the independence of the toxic and antigenic properties of the nucleoprotein obtained from the plague bacillus, but this is not so. The mother substance from which the efficient vaccine is to be obtained must be of a toxic nature towards the animal it is desired to protect, but it is possible by hydrolysis to abate or destroy its toxicity without interfering with its antigenic properties. It would seem as if the antigenic and toxic properties of the nucleoprotein were closely connected—possibly a common molecular grouping is an essential part of both.

In this connection we are reminded of the established relations that exist in the case of diphtheria between toxins and toxoids. Diphtheria toxin on keeping spontaneously degenerates into toxoids. The endotoxine of the plague bacillus does the same, that is if we admit that the atoxic product of the hydrolysis of the toxin is analogous to the toxoid of diphtheria. These analogies are sufficiently apparent, but it should be noted that whereas in the case of plague the toxoid—so to name it—produces in animals not only antitoxines but antibacterial bodies as well, in the case of diphtheria the toxoid, and for that matter the toxin itself, produces only antitoxic substances.

An apparent conversion of a virulent plague bacillus into the bacillus of pseudo-tubercle.

These experiments and the conclusions based thereupon emphasise the close connection existing between the bacillus of plague and that of pseudo-tubercle. The only test capable of distinguishing between the two organisms is an inoculation test. Pseudo-tubercle will not kill rats with the signs and symptoms of plague. To this may perhaps now be added that the nucleoprotein obtained from the pseudo-tubercle bacillus will not immunise rats against plague. But if the animal test fails us, how are we to distinguish between the two organisms? In that case it would appear that there is no means of settling the question. If two organisms from different sources cannot be distinguished by any known test it must for the time being be assumed that the two organisms are identical. If then we can so manipulate the bacillus of plague as to make it conform in its behaviour towards animals in all respects with that of the bacillus of pseudo-tubercle, then we must conclude that the two organisms are fundamentally the same, and relegate the difference in their effect on animals to the category of virulence.

We now proceed to show that by appropriate treatment the differences between a plague organism and the bacillus of pseudo-tubercle as regards their effect on animals (rats and guinea-pigs) can be made to disappear.

Some years' experience in the maintenance of a virulent strain of plague at its full virulence over a long period of subculture has suggested the following procedure. The culture is kept under such conditions as reduce the rate of growth to a minimum, and when it is required for test purposes it is put under such conditions as make the rate of growth a maximum. This amounts to keeping the stock culture cool and immediately before use transferring it to fresh medium at the optimum growing temperature. Any conditions which allow the bacilli to grow in the presence of their own products seem to lower the virulence and *vice versa.*

In conformity with this experience the experiment was tried of growing the stock virulent plague culture in a solution of the nucleoprotein obtained from the plague bacillus itself. Good growth took place. After two days' growth the culture was inoculated into a series of rats and guinea-pigs with the following results:

Ten animals were used in each series. Among the ten rats no acute deaths took place. One died on the fifth day with no signs of plague, three others died on the second, eighth and tenth days respectively. In none of them was there any signs of plague. Of ten control rats inoculated at the same time with the same culture grown in ordinary broth seven died of acute typical plague.

Growing the virulent bacillus in its own nucleoprotein has thus abolished the virulence of the organism as regards rats. The dose of the cultures (0·1 c.c.) administered was the same in both cases[1].

It would seem then that the effect on rats of the inoculation of a culture grown in a solution of its own nucleoprotein is the same as would be produced by the inoculation of a typical culture of pseudo-tubercle, the usual results of such an inoculation being the death of a certain number of the rats without any noteworthy signs. From such inoculated rats it is impossible to recover the pseudo-tubercle bacillus[2].

Now as to the effect on guinea-pigs of the same culture.

Ten guinea-pigs were inoculated with the same dose as the ten rats. Of these ten pigs all died. The days on which they died were the eleventh (three), fifteenth and seventeenth respectively. On the fifteenth day five pigs were so ill that they were killed. All were examined post-mortem. In all the appearances were the same, viz. those typical of pseudo-tubercle. In this opinion Dr MacConkey, who examined the animals, quite agreed.

Ten control guinea-pigs inoculated with the same culture grown in ordinary broth gave the following results: nine died, the times of death being 6, 8, 6, 7, 6, 3, 5, 5, 5 days respectively.

By growing the virulent bacillus in the solution described it appears that not only is the day of death postponed, which is characteristic of pseudo-tubercle, but in addition the post-mortem appearances have changed from those typical of acute plague to those typical of pseudo-tubercle. This latter point must not be pressed too far as it is often impossible to distinguish between the two appearances. A known culture of plague will sometimes kill guinea-pigs with post-mortem signs indistinguishable from those of pseudo-tubercle.

The broad fact remains that in these experiments we have converted the bacillus of plague which kills rats acutely into a bacillus which will hardly kill them at all and then without any of the post-mortem signs of plague. On the other hand as regards guinea-pigs we have converted the acute killing bacillus into a chronic killing bacillus. In fact the bacillus that is found in the culture of the plague bacillus in the nucleoprotein solution is indistinguishable from a typical pseudo-tubercle bacillus.

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[1] Even if the density of the cultures was not so nearly the same as it appeared to be, it has already been shown (vol. x. p. 543) that the fatality does not vary much with the dose within wide limits about $\frac{1}{10}$ c.c.

[2] Personal communication by Dr MacConkey, and Dr Martin tells me he has had the same experience.

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LX. OBSERVATIONS ON THE MECHANISM OF PLAGUE IMMUNITY.

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THE FATE OF THE PLAGUE BACILLUS AFTER INOCULATION INTO RATS.

I. PERITONEAL INOCULATION.

1. *The mechanism in normal animals.*

THE introduction of plague bacilli into the peritoneal cavity is followed by a reaction which is typically inflammatory. The first effect is a secretion of fluid containing cellular elements. The fluid is rich in a fibrin-forming mechanism, and the cells are so numerous as to give the fluid a milky appearance. They are during the first hours principally mononuclears and lymphocytes, the polynuclears appearing in force about the third hour. The cells are actively phagocytic but not uniformly so. It is common to find amongst a mass of polynuclears a minority that are crowded to bursting point with bacilli and a majority that are quite free from bacilli. Examined microscopically (on the warm stage) both are found to be actively amoeboid so that there is no question of killing by, for example, endotoxine. The fact is difficult of explanation.

The fluid, as has been remarked, readily clots. This clotting power forms the basis of a mechanism of great beauty. If a little of the exudate be removed and examined microscopically on the hot stage, delicate fibrils of fibrin are seen to form and to traverse the field in all directions. To these fibrils the bacilli become attached. So marked is this effect that the first evidence of the formation of the fibrils is often the arrangement of the bacilli in lines. A network of fibrils is thus formed traversing the fluid and enclosing in its meshes bacilli and cells. Soon the network, or more aptly the spongework, as it is extensive in all dimensions, begins to contract. The contraction continues until there results a mass of cells, bacilli and fibrin inextricably entangled.

In this way are formed in the peritoneal cavity small flecks floating in the exudate. Many of the cells are full to bursting point with organisms, others are plasmolysed setting free their granules, a few appear healthy. In some cases it appears as if so many cells have coalesced that the bacilli are lying in a syncytium of cells. Prominent in such a mass of cells are the eosinophile granules and the large basophil granules characteristic of the rat. The whole process is, in its first effects, a very perfect netting mechanism, resulting in a close aggregation of cells and bacilli. The effect is to clear the peritoneal cavity of bacilli and to bring them into intimate association with inflammatory cells. In this process the omentum plays a part. The ultimate fate of the flecks as I have called them above is to become attached to the omentum. If sufficiently numerous the omentum becomes retracted and rolled up enclosing masses of the flecks. In time, if the animal survives, the centre of the mass may become an abscess cavity.

In the course of the above process the animal may die at any stage. This death is presumably due to the absorption of endotoxine, for invasion of the body by bacilli has not yet taken place. At any time during the process there are observed bacilli which are free and have escaped the netting, and the fate of the animal depends on the completeness with which the process can accomplish itself. On the one hand we have the process as described progressing in an orderly sequence, on the other we have the bacilli multiplying at a certain rate. The fate of the animal depends on these two rates. If the rate of the process can compete successfully with the rate of multiplication then the animal survives, if on the other hand the rate of multiplication of the bacilli is greater than the mechanisms of netting, lysis and phagocytosis can compete with, then the animal succumbs to plague.

2. *The mechanism in immune animals.*

In an immune animal (*i.e.* an animal that has been vaccinated so that out of 100 animals so vaccinated 80 survive a certain lethal dose of virulent plague) precisely the same course of events takes place. At any stage the differences observed are not differences of kind but of degree. Thus whereas in the normal animal the number of bacilli escaping the process are many, in an immune animal there will be no bacilli outside the net. The extent of the reaction in the normal and immune animals seems to be the same and to depend more on the quantity of bacilli present than on anything else. *The number of bacilli on the other hand in the case of the immune animal is at any stage of the* *process much less than is the number at the same stage in the case of the normal, unvaccinated animal.*

The differences observed in the two cases are all explicable on the assumption that in the case of the immune animal the multiplication of the bacilli is much less than in the case of the normal animal.

II. SUBCUTANEOUS INOCULATION.

As peritoneal infection is hardly a natural mode of infection, it was possible that the conclusions drawn from its study might be erroneous. The usual method of infection in plague is a cutaneous or a subcutaneous inoculation, both in the laboratory or in the natural epidemic. A series of rats were accordingly inoculated subcutaneously with virulent and avirulent plague. A method had to be devised for following the course of events. It was possible to cut series of sections and to study them at leisure. A consideration of the difficulty and labour of getting a comprehensive view of the course of events from some thousands of sections led to its abandonment and the following method was employed. A rat was inoculated subcutaneously, taking care that the dose was deposited close under the skin, so that it might be possible to recover the bacilli and exuded cells from the point of inoculation, and to examine the condition of affairs by the method of smears. This method has the advantage that a living preparation may be put up and examined on the hot stage at any time. It is essential that the bacilli should be deposited in a limited area which can be subsequently identified. This is best ensured by inserting the needle from the opposite side of a limb until its point can be felt pushing up the skin on the other side. About $\frac{1}{10}$ c.c. of culture is then deposited close under the skin at the point selected. The needle is then withdrawn through the muscle of the limb.

When it is desired to examine the course of events the whole skin is reflected including the subcutaneous tissue. The point at which the bacilli were deposited is then recognised through the fat and areolar tissue as a whitish spot. This is opened and smear preparations made and stained, usually with Leishman's stain. Apart from the usual and well known inflammatory reaction which is common to the presence of any foreign body the following specially interesting points were observed.

The difference between the reaction in the case of the normal and the immune rat was, as in the case of the peritoneum, one of degree and not of kind. Whereas in both cases the bacilli increased, in the case of the immune rat the number of bacilli recoverable at any stage of the process was always much less. Concurrently the extent of the reaction in the immune rat is always much less than in the case of the normal rat, the extent of the reaction depending, other things being equal, on the number of bacilli present.

The conclusion reached was that the rate of increase of the bacilli in the normal rat was always much greater than was the rate of increase in the case of the immune rat. This conclusion may be expressed in another way by saying that the normal rat provides a good culture medium for the plague bacillus whereas the immune rat provides a very bad one.

One series of rats examined in this way may be especially alluded to. It was a series inoculated with a strain of plague that had become, on long subculture in the laboratory, almost completely avirulent. The reaction in this case was of the same typical inflammatory character as in the case of the virulent bacillus. Day by day it was possible to examine the condition of affairs for a week. This was possible as the bacillus being avirulent the rats did not die. For as long as four days after the inoculation free bacilli were found at the site of inoculation. A certain amount of phagocytosis had taken place but this was not complete, bacilli were still after this long time slowly multiplying, unattacked by the phagocytic process. Under the same circumstances of inoculation but with a virulent bacillus the rat would in all probability have been dead by the end of the fourth day. There would have been found at the site of inoculation a vast number of bacilli with a correspondingly extensive reaction. This reaction would not have differed in kind from the small reaction observed in the case of the avirulent bacillus. The difference in the two cases impressed on one is that the virulent bacillus can multiply readily in the body of the rat whereas the avirulent organism can only do so exceedingly slowly or not at all.

In the same way a virulent organism multiplies slowly in the body of a vaccinated rat and rapidly in the body of a normal rat. As regards the virulent organism in the body of the rat the effect of vaccination has been to convert the picture into that of an avirulent one in a normal rat.

It is impossible to give a quantitative measure of these experiments but from the examination of a large number of animals the conclusion is forced upon me that *the essential factor in plague immunity is one which affects the multiplication of the bacillus.*

The multiplication of bacilli in rat serum.

We must now examine this question specifically and determine whether there is a difference in the rates of multiplication of plague bacilli in the body fluids of normal and immune rats. It will be convenient to begin with the serum.

Method of investigation. A method is required that will give at any moment the bacilli existing in the serum under examination. Plating was out of the question as it was soon found that the bacilli grew in chains when the medium contained serum, and one could not by plating discriminate between one bacillus and several hundred bacilli under these circumstances. The same applies to a dilution and end point method. The only method available is direct counting under the microscope. This involves the examination of a given volume spread in a layer of uniform thickness and an illumination capable of defining the individual elements distinctly. The first method tried was to use a Thoma counting cell as in the enumeration of blood elements and dark ground illumination with a paraboloid condenser. A difficulty arose owing to the fact that in serum the organisms settled very slowly; under these circumstances it was necessary to count throughout the depth of the cell and when the focus was adjusted to any one point save the bottom, on which the cross lines are engraved, the diffraction rings from these lines obscured the images of the bacilli. In addition the obliquity of the illuminating cone in the case of the paraboloid is so excessive that if the medium was not optically clear the diffracted images of the suspended particles further obscured the images of the bacilli. These difficulties were overcome by having special counting chambers made (by Zeiss) without any rulings. The place of the rulings was taken by two spider lines in the eyepiece. These are adjustable and can be set at any desired interval. They always remain in focus and the entire depth of the cell can be examined and the bacilli at all levels counted.

The difficulty of the obliquity of the illumination was overcome by discarding the paraboloid and using in its place the aplanatic condenser of Zeiss to which was adapted a suitable central stop. Most of the counts were made with the 8 mm. apochromatic objective of Zeiss with a central aperture stop. Some of the counts were made with the 4 mm. of the same series.

The dark ground effect with this arrangement is very perfect. The bacilli stand out as bright rings on a dark ground and no difficulty arises from impurities in the medium, at least these difficulties are minimised. The depth of the cell is constant and known, the width is read off from the screw adjusting the distance apart of the spider lines in the eyepiece, the length is measured by a vernier attached to the mechanical stage. Thus the volume counted over is accurately known, and the number of organisms per unit volume can be expressed in any convenient terms. In what follows the units are millions per cubic centimetre.

If a small number of plague bacilli that have been grown on agar be introduced into fresh normal rat serum, growth as a rule fails to occur. If the fate of the introduced bacilli be watched hour by hour they are seen to gradually lose their bright outline (bright under the conditions of illumination employed) and to finally become indistinguishable. At the same time they lose their power of being stained. If a larger number be introduced the same fate befalls a proportion of them, the remainder after the lapse of some hours beginning to grow in chains. The proportion that begin to grow after surviving the lytic power of the serum is surprisingly small. Thus of 20 million organisms that were placed in fresh normal rat serum, only half a million were found after 10 hours' incubation at 37° C. The recognition of the living organisms under these circumstances is facilitated by counting the number of multiplying units. Thus in the case mentioned 20 million individual organisms were introduced, the bacilli being obtained from the surface of agar and emulsified in salt solution by the aid of shaking with beads until no clumps remained. After 10 hours, multiplication of the survivors in the serum had commenced and small chains of three or four organisms were seen. A count of the number of these chains gives the number of the individuals that survived the lysis in the normal serum. This lytic effect of normal serum is an obstacle in investigating the growth of plague in immune serum.

As we have seen, the plague bacillus contains within its body an endotoxine. On the occurrence of lysis this endotoxine is liberated and if an immune serum is under investigation the liberated endotoxine will neutralise the antibodies present in the serum. Thus the very substances whose presence it is desired to investigate become wholly or partially neutralised at the outset and the maintenance of a clean experiment becomes impossible.

This lytic effect of normal serum is not confined to plague: in typhoid, for example, it constitutes a grave source of inaccuracy in the opsonic technique. The difficulty in this case has been overcome by the introduction of a dilution method. The adoption of such a method in the investigations with plague was not considered advisable as it was desired to investigate the growth of the organism under conditions as closely approximating to those obtaining in the body as possible. Another way out of the difficulty suggested itself. From one point of view the effects of the preliminary lysis may be regarded as breeding out from the agar or broth strain of organisms a serum-resistant race. This point of view is rendered probable from the consideration of some facts as to the numbers of bacilli lysed according to the number introduced into the serum. If the lytic process were one concerned with specific antibodies these would be used up in the process and the introduction of a sufficiently large number of organisms would exhaust them. This is not found to be the case, the number lysed being approximately a function of the number introduced. There is thus a selection among the bacilli, some of them being capable of growing in serum and others not.

On the other hand this lysis in normal serum appears to depend on the presence of fresh complement as it can be abolished by a preliminary heating of the serum. Eight million organisms were introduced into one cubic centimetre of rat serum that had been heated to 55° C. for half an hour, multiplication commenced and at the end of four hours the number had increased to 30 millions. In a parallel experiment with the addition of 0·02 c.c. of fresh rat serum there was no multiplication at the end of four hours.

A method of circumventing the complicating process of lysis in fresh normal serum is as follows. If a certain number of bacilli be introduced into fresh normal serum the survivors begin to multiply in chains which can be recognised at the end of say 10 hours as being of a certain length or composed of a certain number of organisms. The rate of multiplication of the survivors, as indicated by the length of the chains after the expiry of a certain time, is accelerated by the presence of endotoxine. Thus, in a certain experiment 20 million organisms were added to one cubic centimetre of fresh normal rat serum, and at the end of 10 hours the number was reduced to half a million and the survivors had not begun to multiply. In parallel experiments in which varying amounts of endotoxine had been added to a series of tubes containing the same amount of serum and bacilli the results were as follows:

Amount of endotoxine	Appearance of survivors
0 mg.	No chains
0·02 ,,	No chains
0·04 ,,	No chains
0·08 ,,	Chains of four or six
0·16 ,,	Very long chains

A preliminary lysis occurs, after which the rate of growth of the survivors is accelerated by the presence of the endotoxine. The fact that the endotoxine can be added in increasing amount before this effect is noticeable, suggests that a certain proportion is combined with the serum before its accelerating effect becomes apparent. If this experiment be repeated with immune serum the same effect is observed with this important distinction that the amount of endotoxine that must be added to produce the accelerating effect is very much larger. Thus in one experiment with fresh immune rat serum to which 10 million organisms had been added at the expiry of 10 hours the following effects were observed:

Amount of endotoxine	Appearance of survivors
0 mg.	No organisms recognisable.
0·2 ,,	,, ,, ,,
0·4 ,,	0·06 mill. multiplying elements in short chains.
0·8 ,,	0·1 mill. elements in short chains.
1·6 ,,	0·2 mill. elements in long chains.
3·2 ,,	0·7 mill. elements in very long chains.
6·4 ,,	Luxuriant growth in chains and masses too numerous to count.

The appearance in the tube that had received 0·8 mg. in the case of the immune serum was similar to that in the tube that had received 0·08 in the case of the normal serum. These experiments were repeated with similar results. It would appear that the endotoxine of the plague bacillus prepares the serum in some way, rendering it a good culture medium for the organism and that this effect is neutralised by immune serum.

These experiments are incomplete. So far as they have been carried however they give some indication of the mechanism of the immunity conferred by the inoculation of the endotoxine.

The characters of this immunity as evidenced in the suggestive experiment last mentioned give ground for regarding it as an anti-endotoxic immunity. On this view the function of the endotoxine of the plague bacillus is to counteract the disadvantageous effect of some constituent of the serum and to render the rat a good culture medium for the bacillus. How it accomplishes this is not clear; it may well be that the proteolytic enzyme contained in the bacillus and always present in the endotoxine plays a part. If so it must have a very high velocity coefficient. In experiments that have been made to test the anti-endotoxic power of horse serum it apparently makes little difference whether the mixture of toxin and serum is allowed to stand for more or less time before inoculation into the test animal.

Whatever the mechanism, the fact remains that the same toxine that is capable of provoking the formation of antitoxine in the horse is capable of neutralising it in the immune serum of this animal, while when it is injected into rats it provokes an immunity to infection with the living organism, the essential features of which seem to be resident in the serum of this animal, and to be in turn capable of being neutralised by the endotoxine.

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LXI. THE ONSET AND DURATION OF THE IMMUNITY CONSEQUENT ON THE INOCULATION OF PLAGUE NUCLEOPROTEIN.

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It has been shown in a previous report (vol. x. p. 559) that consequent on the inoculation of the nucleoprotein obtained from the plague bacillus an immunity is established to the subsequent inoculation of living plague bacilli in broth culture. Some indication of the extent of this immunity was obtained by comparing the mortality of the inoculated rats after the test inoculation with the mortality that occurred (78 %) in uninoculated rats. In all these investigations the time that was allowed to elapse between the protective inoculation and the test inoculation was 14 days. We have now to consider the influence of the length of time elapsing between the protective and test inoculations upon the protection.

It has also been shown that the extent of the protection consequent on the inoculation of the nucleoprotein is of the same order in the cases where this substance is employed as a vaccine either in its fresh toxic condition or in its autolysed and relatively atoxic condition (vol. XI. supplement, p. 38). These two conditions of the nucleoprotein will also be considered in relation to the onset of immunity.

ONSET OF THE IMMUNITY.

I. *Immunity conferred on rats by inoculation of the nucleoprotein immediately after, before or simultaneously with the test inoculation.*

A. Using fresh toxic nucleoprotein in 0·01 mg. doses:

of 10 rats inoculated 4 hours before the test dose 7 died 3 survived
,, ,, ,, at the same time as the test dose 6 died 4 survived
,, ,, ,, 5 hours after the test dose 7 died 3 survived
,, ,, ,, 18 ,, ,, ,, ,, ,,

B. Using 2 months autolysed nucleoprotein in 0·01 mg. doses:—

of 10 rats inoculated 4 hours before the test dose 6 died 4 survived
,, ,, ,, at the same time as the test dose 8 died 2 survived
,, ,, ,, 5 hours after the test dose 8 died 2 survived
,, ,, ,, 18 ,, ,, ,, ,, ,,

From these experiments the conclusion is justified that the inoculation of the nucleoprotein either in its toxic or atoxic condition is without effect when the time that elapses between its administration and the test dose is less than 18 hours.

II. *What is the earliest time after the inoculation of the nucleoprotein at which evidence of protection against living infection is noticeable?*

A. Using toxic nucleoprotein in 0·01 mg. doses, *i.e.* $\frac{1}{10}$ lethal dose:

1 day	after the inoculation	8 died	16 survived,	*i.e.*	66 %	survived
2 days	,, ,,	2 ,,	18 ,,	,,	88	,,
3 ,,	,, ,,	0 ,,	17 ,,	,,	100	,,
4 ,,	,, ,,	2 ,,	17 ,,	,,	89	,,
14 ,,	,, ,,	2 ,,	17 ,,	,,	89	,,

B. Using the relatively atoxic nucleoprotein in 0·01 mg. doses, *i.e.* $\frac{1}{100}$ lethal dose:

1 day	after the inoculation	8 died	11 survived,	*i.e.*	60 %	survived
2 days	,, ,,	7 ,,	11 ,,	,,	72	,,
3 ,,	,, ,,	3 ,,	14 ,,	,,	88	,,
4 ,,	,, ,,	4 ,,	15 ,,	,,	84	,,
14 ,,	,, ,,	2 ,,	12 ,,	,,	83	,,

The results of these experiments may be put in the form of a curve when their meaning becomes more evident.

From these curves the following conclusions are justified:

1. That after the inoculation of nucleoprotein in 0·01 mg. doses the development of the resulting immunity is very rapid, being distinctly evident 24 hours after vaccination[1].

2. The highest point on the curve, *i.e.* the greatest immunity, occurs on the third day.

3. That a slightly better immunity is evident on the third day after the inoculation of the more toxic vaccine.

The conclusion that for the rapid production of immunity it is advantageous to employ a toxic vaccine is capable of control in the following way. In previous reports it was pointed out (vol. XI. supplement, p. 36) that the toxicity of the nucleoprotein was greater the shorter the time elapsing since its extraction from the bacillus, and that there was reason to suppose that as it existed within the body of the bacillus its toxicity was at a maximum. Consequently if there is any relation between the toxicity and the rapidity of onset of the immunity, the vaccination by the same dose of nucleoprotein wrapped up in the bodies of the bacilli should be followed by a still earlier development of the maximum immunity. Using such doses of whole bacilli killed by heat (about 0·1 mg.) as contained 0·01 mg. of nucleoprotein, the following results were obtained:

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[1] Working with Haffkine's prophylactic as an immunising dose, wild Indian *Mus rattus* and an emulsion of a plague rat's spleen as an infecting dose, Stevenson and Kapadia (*Report of the Bombay Bacteriological Laboratory for* 1911, p. 26) found evidence of some protection as soon as 8½ hours after vaccination.

1 day	**after the inoculation**	**18 survived**	**1 died,**	***i.e.***	**95 $^0/_0$ survived**
2 days	,, ,,	16 ,,	3 ..	.,	84 ,,
3 ,,	,, ,,	14 ,,	3 ,,	..	82 ,,

These results are plotted in figure 1.

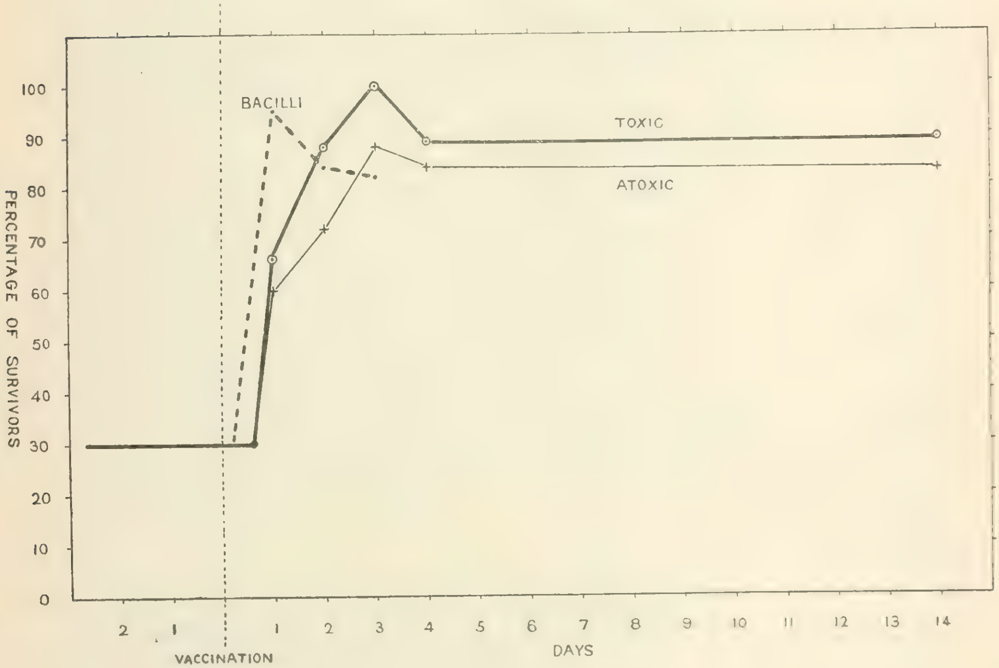

Fig. 1.

Considering the three curves together it will be seen that the highest point is reached first in the case of the whole bacillus vaccine, that is in the case of the vaccine in which we have reason to suppose that the contained nucleoprotein is in the most toxic condition. The conclusion was previously (vol. XI. supplement, p. 42) reached that the protective and toxic properties of the nucleoprotein contained in the plague bacillus were distinct. This conclusion was based on the consideration of the results of vaccination of rats when the interval between the vaccinating and the test dose was 14 days. At this time it remains true that there is no discoverable difference in the results of the two (toxic and relatively atoxic) vaccines. But in the earlier stages of the onset of immunity a difference emerges. With the more toxic vaccine the onset of immunity is slightly more rapid; this early rise however appears to be associated with a corresponding early fall so that at the end of a fortnight there is no difference in the height of the immunity curve.

An observation of some interest was made in connection with the series of rats last quoted. These rats were inoculated (vaccinated) with whole bacilli that had been heated to 60° C. for half an hour. The resulting immunity was completely established as early as the first day. With a view to determining the fate of the bacilli used as the vaccine, certain of the rats were kept and the site of vaccination examined from day to day. There was no difficulty in recognising the presence of the bodies of these bacilli. Now it has been repeatedly shown that all the immunity that a culture of plague is capable of conferring on rats is represented in the nucleoprotein extract obtained from them by the methods described in these reports, the rest of the bacilli being incapable of conferring any immunity. In conformity with this, in the series under consideration the bodies of the bacilli were recognisable microscopically up to the fourth day after the inoculation, that is three days after the establishment of all the immunity that the vaccinating dose of organisms is capable of conferring. The resulting immunity must therefore have depended on the escape of the contents of the bacilli, the presence of any unabsorbed portion being hardly capable of being considered responsible.

THE DURATION OF THE IMMUNITY.

Already, on the fourth day after the vaccinating dose of nucleoprotein the differences observed in the height of the immunity curve according as fresh or autolysed nucleoprotein has been used for the vaccination are seen to be very small. After the expiration of 14 days these differences are no longer significant.

It is noteworthy however that the height of the curve remains constant after the slight drop between the third and fourth day, that is to say that the immunity once established remains at the same level. It will now be shown that this level is maintained for some months. The three curves being so nearly coincident at the expiry of 14 days the question of the differences in the extent of the immunity following the inoculation of the nucleoprotein in its fresh or autolysed condition will not be further considered.

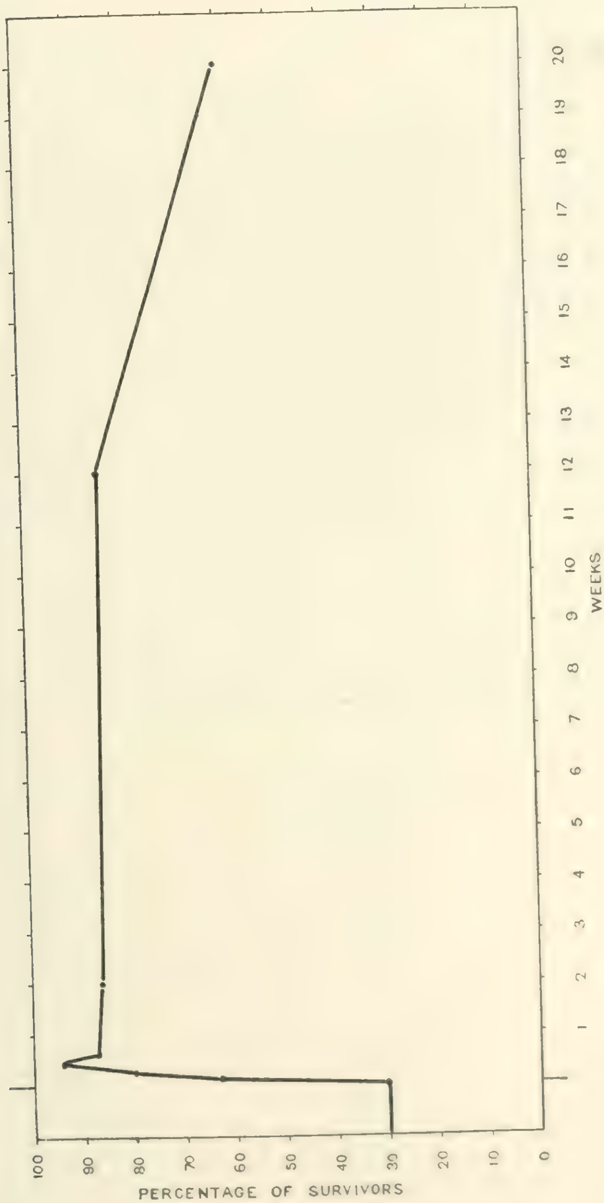
WEEKS
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
PERCENTAGE OF SURVIVORS
100
90
80
70
60
50
40
30
20
10
0

Fig. 2.

In connection with these experiments considerable difficulty has been encountered in keeping rats alive and healthy for long periods of time. It was hoped that the curve of immunity might be continued for a year at least. On two occasions some hundreds of rats were vaccinated and provision made for keeping them for a long period. This has not been found possible. Epidemics of various diseases broke out during the hot summer of 1911, and after the disappointing loss of much valuable material it has only been found possible to obtain reliable data up to five months after the vaccination (see fig. 2).

At three months after vaccination a series that received 0·1 mg. and one that received 0·01 mg. are available. Of the first series every one of 19 rats survived the test inoculation. Of the second 85 °/₀ survived, the series comprising 20 rats. The immunity thus lasts unimpaired for three months at the same height as it was at the end of 14 days.

At five months only one series of 31 rats was available. Of these 18 survived the test dose, *i.e.* 60 °/₀, from which it would appear that in rats the immunity after a single dose of the nucleoprotein is still very appreciable after five months though a good deal less than after three months.

It need hardly be pointed out that the times ascertained in these experiments for the onset and duration of immunity in the rat cannot be directly transferred to any other species of animal, especially when the size and rate of metabolism are widely different from those obtaining in the rat.

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On the Organisms of the Typhoid-Colon Group[1] and their Differentiation.

[Lister Institute, London.]

By **J. Henderson Smith.**

I.

In the year 1906 M. Neisser recorded the occurrence of a coliform organism, which while primarily a non-fermenter of lactose, yet could give rise to a fermenting strain when grown on lactose-agar. This organism was very carefully and thoroughly studied by Massini, who proved that the fermenting strain was not a contamination but was really derived from the original non-fermenting organism and arose upon lactose-agar as a variant with a new character, which bred true. Since then numerous instances have been recorded by Burk, Jacobson, Revis, and others and in particular by Twort and Reiner Müller, of members of the Colon-Typhoid group, which displayed a similar capacity of varying towards one more of the carbohydrates, and the fact of bacterial variation in this direction is now beyond dispute. The important bearing which this fact may have upon the differentiation and identification of the pathogenic intestinal organisms is obvious, and for the last year or two W. J. Penfold has been carrying out in the Lister Institute a series of investigations on the subject. It is largely on his work that the following considerations are based.

If a broth or peptone-water culture of a normal typhoid bacillus be planted on the surface of a dulcite-agar plate, to which neutral red has been added, the colonies which develop after incubation are all without exception colourless. They do not produce enough free acid to turn the colour of the neutral red, and it is reasonable to suppose that they are composed, at least for the most part, of individuals which do not ferment the alcohol. When we continue to observe such a dulcite-agar plate, on which the colonies are discrete enough to allow of their free development, on a considerable number of the colonies papillae begin to appear, after a period which varies in different cases from 3 to 10 days. These papillae are small very definite protuberances of characteristic appearance, somewhat irregular in shape and of variable size. Their number per colony, as also the number of colonies which show them, varies considerably, but the number both of papillae and of papillated colonies usually increases as time goes on. Eventually a proportion of the papillae turn red. On these plate cultures of typhoid, then, the organisms, which at first failed entirely to show any signs of fermenting dulcite, now do so, but only in certain limited parts of the total growth. It might appear that what is occurring on the plates is that the organisms are turning to the dulcite as a source of food, having first exhausted the accessible supply of other constituents of the nutrient agar. In other words that typhoid bacilli are capable of fermenting dulcite, but refuse to do so in the presence of other more appropriate nutriment. This is not the explanation of the late fermentation. If the organisms which compose such a papilla are plated out again upon dulcite agar, a number of the colonies which appear are red throughout, and these show no papillae. There is no longer an interval of several days, during which other food supply is being used up, but the fermentation begins early. The papilla then contains bacilli, which unlike normal typhoid are capable of attacking dulcite from the first. The papillated colony arose from a single organism of the original culture, and its progeny were at first, if not all, at all events preponderatingly non-fermenting. From among this progeny has arisen a strain, which now yields descendants preponderatingly fermenting. This new character ist at first unstable, and after a short period of growth on normal media, the fermenting strain will again give progeny all or nearly all non-fermenting. But continued selection of colonies from dulcite agar or a few months training on dulcite broth establishes the character, and a strain is obtained which remains a dulcite-fermenter, even after many months of cultivation in the absence of dulcite.

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[1] At the Berlin meeting of the International Congress of Hygiene and Demography in 1907 certain Institutes were requested to untertake an inquiry into the differentiation of the "typhoid-like" bacteria, and to present a report upon these organisms at the Washington meeting in 1912. This paper constitutes the report of the Lister Institute of Preventive Medicine, London.

The sequence of events is perhaps more easily followed on plates, but a similar process takes place in dulcite-broth. All or nearly all normal typhoid strains eventually turn a dulcite broth acid, but the time taken to do so is very variable varying in Penfold's strains from 5 to 15 days. This time can readily be reduced by continued culture in dulcite broth to three days, and eventually to one day. By following the changes which occur in litmus dulcite peptone-water, inoculated with normal typhoid, Penfold has shown the sequence of events leading up to the eventual acidity of the medium. Multiplication at first occurs just as in peptone-water containing none of the alcohol, and reaches the maximum of some 200 millions per 1 cc, after which there is a slight decrease. During this period plating on dulcite-agar shows that the organisms are non-fermenters. Then there develops a second rapid and very large increase in the number of organisms which may reach 600 to 1000 millions per 1 cc. This is entirely due to the development of dulcite-fermenting organisms, and the medium turns acid. The non-fermenting individuals almost completely disappear, and it appears almost as if the two strains were antagonistic to one another. If as occurs in one type of culture the numbers do not show this second great increase, the non-fermenters remain permanently in the majority. Fermenting individuals appear but do not gain the upper hand, and the medium does not turn the full deep red of marked acidity.

So far as is known at present, growth on dulcite is the only means by which a dulcite fermenting strain can be obtained. This naturally suggests that the dulcite acts directly upon the individual typhoid bacillus in some specific manner, causing it to produce fermenting variants, which do not arise without this specific stimulus. This may eventually prove to be the correct view, but at present there is little to support it, and it is difficult to reconcile with some facts already known. If a non-fermenting individual be taken from a tube in which fermenting organisms have already arisen, it takes as long to produce a fermenting variant as an entirely untrained organism. It shows no indication of having been affected in any way by the dulcite, and there is nothing to suggest that it has been partially trained. The facts point rather to another interpretation, which is sufficient to account for them without the hypothesis of specific alteration. It is enough to suppose that the normal typhoid bacillus, even growing on a broth medium without dulcite, tends to produce variants which have to a greater or les degree the power of fermenting the alcohol. On a non-dulcite medium there is nothing to encourage this type as compared with others, and as on its first appearance it does not breed true and occurs in very small numbers, the variant disappears again. But if there is dulcite present, such organisms as are able to attack it receive a stimulus to their multiplication which the non-fermenters do not receive, and increasing to a disproportionate extent may eventually displace them entirely. The effect of the carbohydrate in the medium is to select one type of variant amongst those normally occurring, rather than to induce the production of a quite new type. This increase of growth in the presence of an attackable carbohydrate is, as we have seen, what occurs in dulcite tubes, when the fermenting variety appears, and it holds with other sugars also. A medium containing glucose, for example, supports and produces a larger population of typhoid, than one which contains lactose or cane-sugar, which the organism cannot attack, and in which the numbers are approximately the same as when no sugar is present. If the typhoid has been previously "trained" to attack lactose, then the numbers exceed the normal and approach those of the dulcite-variant or glucose populations. The development of a protuberant papilla of itself indicates a greater energy of growth on the part of its components than that of the rest of the colony.

We have here then an instance of variability in the typhoid bacillus such that within a comparatively short time two races can be obtained differing from one another in the definite property of fermenting dulcite. Several other variations in the properties of typhoid with regard to carbohydrates are known. Reiner Müller has described the appearance of papillae on isodulcite, and Penfold like him has found this appearance of papillae to be a constant character of typhoid. If isodulcite plates are made from these papillae, colonies are obtained which show no papillae, and a race is obtained at once, which breeds true and has lost the property of papilla-formation upon isodulcite. An arabinose-fermenting strain is readily obtained, and strains are known which do and others which do not ferment glycerin. Special interest attaches to the lactose-fermenting variety produced by Twort. This organism was subsequently examined in detail by Penfold, and was found by him to be a genuine typhoid both in its lactose and non-lactose states, although it shows one or two slight divergences from the perfectly typical B. typhosus. It took two years constant growth on lactose before the variant could be obtained, and even now after 2 years more on lactose it does not breed true, showing a strong tendency to throw out atavists and revert to its non-fermenting state. After 18 months of training of a considerable number of strains on lactose media, Penfold has failed to produce another fermenting variety of B. typhosus, and it is evident that it is only with great difficulty and after a very long growth on lactose that this particular variant can be produced from normal typhoid. Similar ill-success has attended the attempt to produce variants by growth on a number of other media, such as adonite, erythrite, saccharose, amygdalin, salicin and formates.

It appears then that B. typhosus is an organism which can be made to alter its character with regard to many of the carbohydrates. But it appears also that the readiness with which variation occurs differs in different cases. On the one hand we have the isodulcite-papilla variant, which arises with every strain very rapidly and has the new character fixed immediately. Intermediate comes the dulcite-variant which can be obtained from most strains fairly easily and can be fixed comparatively quickly. On the other hand we have the lactose-fermenting variant, which can be obtained only after years of "training" and has not yet been got to breed true. It may be that even those characters, which have not yet been found to vary are really characters whose variants take exceptionally long to arise, and that there is no single carbohydrate character which may not vary at one time or another under particular conditions. However that may be, the readiness with which the variant appears and the ease with which it becomes fixed show in general a rough agreement, and the more disposed an organism is to produce a particular variant, the more readily is it disposed to take on the new character permanently.

It is probable that when other organisms have been examined in sufficient detail, it will be found that they are like typhoid in this readiness to produce particular variants and reluctance to produce others. Unfortunately the data on this point are still somewhat meagre. The occurrence of variants has been described for many members of the Typhoid-Colon group, among the pathogenic as well as the so-called non-pathogenic organisms occurring in the gut. Thus Reiner Müller has obtained papillae with Paratyphoid B. on raffinose, and with Flexner's dysentery bacillus on isodulcite. Twort has induced the paratyphoids, and also Flexner and Shiga to ferment cane-sugar, and Shiga has also been made to ferment lactose. Hiss so early as 1904 obtained a maltose-fermenting variant of Bacillus Y, and other variations have been recorded. It is a pity that it is not always possible to ascertain from the published accounts how soon these alterations of character arose nor whether the variants bred true, and in some cases the identity of the new strain with the old has not been very satisfactorily established. But it is clear that these variants show great differences in the readiness with which they arise. Massini's lactose-fermenting strain of B. coli mutabile, for example, arises at once and breeds true, while others require weeks or months of training on the particular sugar before a variant appears. More information is much needed on this point, for so far as present knowledge goes it seems likely that along this line is to be found the theoretical justification of the recognised practical value of the carbohydrate tests.

It has been asserted that the possibility of variation is sufficient to destroy the value of the carbohydrate tests as aids to the differentiation and identification of the organisms of this group. If it is possible, so the argument runs, to make Shiga's dysentery bacillus ferment both lactose and cane-sugar, we cannot accept the absence of these properties as significant characteristics of the organism. If plates made from a suspected water or faeces from a case of enteritis show only lactose-fermenting colonies, we can never be sure that these colonies, or some of them, are not really colonies of a typhoid variant. May not something of this kind be the real explanation of the extreme difficulty of isolating typhoid from water?

The difficulty must be admitted, and the possibility recognised that, so far as carbohydrate tests go, one organism may be convertible into another. We can in fact conceive the possibility of an organism with the sugar-reactions of a B. coli and the agglutinating and other properties of a dysentery bacillus, including its pathogenicity. It may even be the case that in certain instances something of this sort does actually occur in nature. Penfold has brought forward some evidence to show that, parallel to the main group of lactose-fermenting organisms in the intestine, there are corresponding groups of non-lactose fermenting bacteria, occurring naturally in fair numbers, which belong to the mutabile class and are convertible into the fermenting types, and he considers that one group may arise from its corresponding group under natural conditions. Even if this were proved, however, beyond dispute, it would not discredit the usefulness of the carbohydrate tests. It only shows us that there are certain organisms for which the lactose relation is unsuitable as a group-character; it does not touch the possibility that there are other carbohydrates more suitable. These particular organisms of the mutabile group have a pronounced tendency to vary towards lactose, and this experience in the laboratory would lead us to anticipate the occurrence of such variants in nature. In a similar way we know that typhoid has a pronounced tendency to vary towards glycerin, and this laboratory experience would lead us to reject glycerin fermentation as a safe character of the typhoid group[1]). The dulcite-fermenting variety is less likely to arise naturally, but even this is a frequently occurring variation with a tendency to breed true. We should prefer to take as a type-character one toward which a variant arises only with great difficulty and breeds true only in very exceptional circumstances existing over a long period, such as the lactose-relation. Lactose therefore is a suitable sugar in the case of typhoid, but unsuitable in the case of B. coli mutabile. Further experience will show for the different groups of organisms what are the carbohydrates on which reliance is to be placed as distinctive media.

To some extent this knowledge has been already acquired. It is usual to make the carbohydrate tests in fluid media with an incubation of several days, say from 3 to 7 days. A variant which does not arise in that time is probably not a very common variant, and so in practice our tests indicate approximately the lines along which further knowledge will be found to develop. The discrepancies which different observers occasionally record in the reactions of organisms (e. g. the typhoid-relation to dulcite, and the different behaviour on solid and fluid media) find their explanation in this occurrence of variations.

It is not claimed by the supporters of the carbohydrate reactions that they are capable of giving a complete and systematic classification even of the intestinal bacteria according to their biological relationships. We have no means of doing this at present with certainty. It must be remembered that most bacteria are extremely variable in other characters as well as in their sugar reactions. In pigment production, toxin-production, gelatin liquefaction and in practically all cultural reactions variability is very frequent. Variations of virulence are amongst the oldest and commonest observations of bacteriology. Even agglutinability is not a constant character, as the frequent occurrence of strains non-agglutinating at the time of isolation from the body is

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1) The converse is not necessarily true, however, since all strains of typhoid produce papillae on isodulcite, but lose this character readily, and no strain has yet been isolated, which is already in the non-papilla-forming state.

sufficient to prove. In many of these instances the variation consists simply of the gain or loss of a definite property found only with the particular species, and not in the acquirement of a character possessed by another and different species, and in this respect such changes may appear to differ fundamentally from the carbohydrate changes. (This may be not a very real distinction, since the carbohydrate reactions are none of them specific under natural conditions in the way in which say the toxin production of diphtheria is specific, but each is common to many species.) Such instances show that variability is one of the characteristic properties of bacterial protoplasm, and that systematic classification is likely to prove exceedingly complex. The carbohydrates do in large measure supply a simple method of practical differentiation of proved value, and it is the only method at present available sufficiently elastic to cover a large number of different organisms. That it does in some instances group together organisms with close pathological relationship is undeniable, and there would seem to be no reason to doubt that as a general rule closely allied strains will produce similar ferments.

It has been suggested by Reiner Müller that variability in certain directions may be actually specific for certain organisms and he instances the case of typhoid, of which all strains give the isodulcite variations. This may be a usual or practically constant character of typhoid, but as he himself recognises, it belongs to other organisms also, and there is no known variation which is quite specific in the sense that it is given by every member of a species, and by no others. A tendency to variation in one particular direction may, indeed, be on occasion a useful aid in differentiating closely similar bacteria. R. Müller found that Paratyphoid B produces papillae on raffinose, while B. Aertryck does not. It is usual on the continent of Europe to consider these organisms as strictly identical. Boycott, Bainbridge and others in England hold that they are distinct, separable by means of agglutinin-absorption tests, and it is an interesting confirmation of this view that Penfold, using the raffinose test, was able to separate a considerable number of strains into two groups, which practically coincided with the groups distinguished by Bainbridge using the absorption method.

In the variations we have been discussing the new strain differs from its parent in a single character; alteration in one character does not necessarily involve alteration in another. Two or more such variations may be superadded, but several characters do not vary simultaneously. Since the fermentation of the different carbohydrates is presumably due to the action of different ferments, and since we have no reason to suppose that power on the part of an organism to produce any one ferment entails either the capacity or incapacity to produce any other ferment, there does not appear to be at present any ground for anticipating that such concomitant variations will often be found to occur. Penfold's experiments on gas-production, however, show that loss of a single ferment may affect several reactions[1]). By growing various organisms on chloracetic acid agar and selecting colonies from the plates, Penfold succeeded in a number of instances in suppressing the formation of gas from sugars, although power to produce acid remained unimpaired. By this means he has bred strains of B. enteritidis Gärtner, B. paratyphosus B, B. Grünthal, and B. coli Escherich (B. acidi lactici and some others remain refractory) which no longer produce gas from glucose. With the loss of this power went the loss of the gas-production from various other carbohydrates. The extent of this loss differs a little in the case of different organisms, but speaking generally it was found that if an organism failed to produce gas from a hexose or pentose which it was able to attack, then it also failed to produce gas from other fermentable hexoses or pentoses, but might continue to produce gas from such alcohols as it was able to attack. The obvious interpretation of this phenomenon is that similar sugars yield as the result of fermentation similar products, which are attackable by a single ferment, but that alcohols yield different products requiring other ferments for their further decomposition. This work of Penfold has another interest in that the altered strains are apparently also the strains which show exceptional powers of resistance to the monochloracetic acid, but the possible significance of this observation cannot yet be fully estimated.

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[1]) With this may be compared the non-gas producing B. Aertryck recorded by **Bainbridge**.

II.

It is usual to divide the bacilli of the Colon-Typhoid group into two main subdivisions according as they do or do not ferment lactose This practice has the sanction of a very large experience, but it must be remembered that the nonlactose fermenters include one group of organisms (that of the B. coli mutabile), which are really more closely allied to the lactose-fermenters. These organisms, which are more common than Massini's experience led him to believe have a pronounced tendency to produce fermenting variants, and in fluid media most of them eventually ferment lactose, though they do so only late, rarely before the sixth day of growth. If the existence of this group is kept in mind little practical inconvenience is caused, and the classification by lactose has the advantage of bringing together the more definitely pathogenic organisms of the intestine hitherto recognised, and is not invalidated by the facts of variation so far as they are yet known. None of the organisms of this division, to which most importance is attached in human pathology, shows any marked tendency to produce lactose-fermenting strains, and in laboratory experience it is only by very long training that the occurrence of fermenting variants can be demonstrated. Further, various workers who have had large experience in this field have the impression that non-lactose fermenters are in some way associated with abnormal conditions of the gut. They occur of course in the normal intestine, but in diarrhoeal or local inflammatory conditions they appear to increase in numbers relatively to the lactose-fermenters. One may recall in this connection the enormous predominance of typhoid and dysentery bacilli which is frequently met with in the stools of carriers or patients suffering from these diseases.

When the group of late fermenters and the gelatin liquefiers have been separated out, there remains a large number of non-lactose bacilli, and amongst these one can distinguish by cultural methods alone three main groups, about which something requires to be said. These three groups are the typhoid, paratyphoid-Gärtner or Enteritis, and the dysentery group, and around these may be collected a large number of organisms, which more or less resemble them. There remain still a number of bacilli, which may be similarly brought together into groups ings of allied members. It is quite probable that some of them have definite pathological importance, but this has not yet been clearly established for any of them. These have been very carefully studied by H. de R. Morgan, who over a series of years examined the intestinal bacilli which neither ferment lactose nor liquefy gelatin. His investigations were concerned chiefly with summer diarrhoea, or cholera infantum (where he found a remarkable associations of this disease with one particular organism rarely occurring elsewhere, and now widely known as Morgan's No. I), but they included also the intestinal flora of children, adults and animals both in the normal state and in other abnormal conditions accompanied by diarrhoea or enteritis. The detailed account of these organisms is to be found in his papers, and a considerable mass of confirmatory and additional information has been accumulated by other workers in cognate and more specialised fields of research.

The Typhoid Group. The typhoid group requires only a brief reference. The characters of the typhoid bacillus are wellknown, and I need only recall here that it produces acid without gas from glucose, mannite and sorbite, turns milk acid without clotting it, and does not ferment dulcite, saccharose nor of course lactose, and does not produce indol nor liquefy gelatin. These characters are constant for all naturally occurring strains of typhoid, with the possible exception of dulcite already discussed. The B. typhosus is in fact a remarkably well defined and stable organism. Very many strains have been examined in a series of carbohydrates much larger than the above without the detection of differences. An exception may be found in glycerin, since both fermenting and non-fermenting strains have been described, and it is probable that glycerin resembles dulcite or isodulcite in being a substance to which variants are readily produced. But it seems beyond doubt that unless artificial means are taken to alter it the typhoid bacillus is very constant in its cultural characters. All these strains, moreover, agree in their serological characters, and there is rarely any difficulty in identifying B. typhosus when it is met. Further, it is an organism to which close allies as judged by the cultural reactions do not occur naturally. (A somewhat similar bacillus has been found by Ledingham to occur frequently in flies, but it produces indol, produces alkali in milk, and does not ferment sorbite.) Non-motile strains have been described and strains inagglutinable on first isolation are comparatively frequent. If we accept, as most would, motility and agglutinability as definite characters of the B. typhosus, such strains must be accepted as subvariants, and they illustrate the necessity of employing more than one criterion in identifying any organism. No one would refuse to accept as typhoid an organism which otherwise typical is at first inagglutinable but subsequently conforms to type after a short period of laboratory culture. But the duration of inagglutinability in actually observed cases is very variable, and may extend to several subcultures. Moreover, other organisms notably Gärtner's Bacillus and Paratyphosus B, are occasionally no less strongly agglutinated by typhoid sera than a true B. typhosus. Neither the presence nor the absence of the agglutination character is in itself finally conclusive, and we must recognise that agglutinability is also a variable characteristic. In the case of typhoid, the stability of the cultural reactions and the non-occurrence of organisms which closely resemble it make these reactions of especial value in the differentiation.

The Enteritis or Gärtner-Paratyphoid Group.

The second group is the Enteritis or Gärtner-Paratyphoid group, of which culturally the type-member is the B. Gärtner. Its chief cultural characters, which are the same as those of Paratyphosus B and B. suipestifer are the following. It produces acid with gas from glucose, mannite, dulcite and sorbite, does not ferment cane-sugar nor lactose, does not produce indol nor liquefy gelatin, and litmus-milk is at first made acid but from about the third day on the milk turns alkaline, and by the seventh day becomes strongly alkaline without at any time clot-formation or digestion. Certain other reactions will be referred to immediately. This group has been the subject of much confusion and is still the cause of a good deal of controversy. To some extent this is a relic of the pre-agglutination days, but to a still greater degree it is due to a loose application of the term "paratyphoid" to imperfectly examined organisms. The cultural characters which we have detailed mark of a definite group of bacille within which further distinction can be made by other methods, as will presently be described. But there occur in the normal intestine several organisms which are culturally very much like the type organisms of the enteritis group but are to be definitely distinguished from them. Of these there are at least two main subgroups, one which ferments cane-sugar and one which does not ferment dulcite. Both are comparatively common, and neither has any claim to the name of paratyphoid. Bainbridge and O'Brien examined a number of strains of the saccharose-fermenters, which had been isolated by Ledingham, and while they found minor variations in the cultural reactions, they obtained no significant agglutination of any of them with Gärtner, Paratyphoid B or other sera made from organisms of the enteritis group. In this their experience agrees with that of Savage and of Morgan, who also found the same to be true of the dulcite non-fermenters. The members of both these subgroups are apparently quite distinct from the genuine enteritis organisms. Unfortunately, however, many workers especially on the Continent of Europe adopt even at the present time criteria which do not exclude these bacteria, and by omitting dulcite, sometimes even saccharose from their tests, they extend the name of paratyphoid to organisms, which certainly should not receive it. This has led to confusion in at least two directions. On the one hand the epidemiology of paratyphoid fever is obscured by their inclusion in this group, and on the other the agglutination test is discredited because so many so-called "paratyphoid" strains fail to give it.

These two subgroups, the cane-sugar fermenters and dulcite non-fermenters are not known to have any pathogenic significance for man, but the pathogenic B. paratyphosus A bears a close resemblance culturally to the type-organisms. Paratyphoid A differs from the latter only in not producing enough alkali in litmus-milk to overcome again the initial acidity it causes. The milk turns acid and remains so permanently without formation of clot or true digestion. This organism can be readily identified by agglutination as well as by the milk reaction. It is not common in Europe, but in India is frequently met with and is a common cause there of socalled "paratyphoid" fever. It is not yet known whether subvarieties of this organism might be distinguished by means of agglutination or other methods, but it may be noted that Morgan has isolated an organism identical in all respects with Paratyphoid A, except that it was not agglutinated by a strong Paratyphoid A serum.

These is still one other group of organisms which can be distinguished from the true type-organisms by cultural tests, if we extend them a little further. The type-bacilli ferment galactose, arabinose and maltose, but produce no apparent change in salicin, raffinose or inulin. In the intestine of healthy animals occurs an organism which resembling the type in other cultural respects differs from it in fermenting salicin. It is fairly frequent, e. g. Savage found it eight times in the examination of 31 animals, and its differentiation is confirmed by the absence of pathogenicity and of agglutination with standard sera.

When the field has been narrowed by the exclusion of these three subgroups, there remains the mass of organisms, which are culturally indistinguishable from the types. As was first shown by Durham and de Nobele, these are separable into two very definite subgroups by agglutination reactions. First comes B. Gärtner, an organism sharply and readily differentiated by means of agglutination, since it is not affected to any significant extent by sera of other members of the group. The fact that it may be agglutinated by some typhoid sera, and that conversely typhoid bacilli may be agglutinated by Gärtner sera, causes no difficulty in identification, because of the other charateristics of these organisms. B. Gärtner is the organism responsible for many attacks of food poisoning, and it is contained in many rat-viruses including "B. Danysz". It is believed to have caused, and is certainly associated with, spontaneous outbreaks of disease in mice, guinea pigs (Bainbridge and O'Brien, MacConkey) and rats (Boycott), and there is some evidence that it is a normal inhabitant of rats and mice, or at all events is apt to develop in these animals when they suffer from depressing or toxic influences. What are the conditions which bring about the sudden epizootic virulence of the organism are unknown, but it is of interest to note that in a similar epizootic in guinea pigs associated with B. suipestifer, Petrie and O'Brien found reason to believe in the occurrence of a filter-passing virus. In a rat epizootic Boycott isolated an organism identical with B. Gärtner in all respects, cultural and serological (including absorption tests), which possessed the property of changing the haemoglobin in infected animals into methaemoglobin, so that their blood was chocolate or brown in colour during life.

The second subgroup, into which serum-reactions divide the culturally typical organisms, in its turn comprises two members, the B. paratyphosus B and the B. suipestifer (with the latter of which authorities are agreed that B. Aertryck is identical). Both organisms are agglutinated by the same sera and to approximately equal degree in most cases. Many authorities maintain that there is no real ground for drawing any distinction between them. Most of the English workers, however, and some German writers believe that they are separable from one another by absorption tests carefully carried out and that they are definitely distinct organisms. Bainbridge and O'Brien, the most recent English investigators to make an extended study of the subject, examined by this test a large number of strains from various sources, and they found that they fell into two groups which corresponded on the one hand to the B. paratyphosus B associated with paratyphoid infection in man and on the other to B. suipestifer, associated with food-poisoning. Their results are so consistent and clear that it is difficult to believe the distinction to be unreal. It has further received support from Penfold's application to their strains of the raffinose-papilla test already described and also from complement-deviation experiments of H. R. Dean.

The culturally typical organisms of the enteritis group then, are divisible into three subgroups, B. Gärtner, Paratyphosus B and B. suipestifer (or Aertryck), and into one or other of these groups fall all or nearly all the organisms associated with food-poisoning outbreaks recorded in many places since Gärtner's original description, as well as many of the organisms associated with animal epizootics. It may be noted here that the B. typhi murium is apparently not a separate species, but of the organisms passing under that name some are B. Gärtner and some are B. suipestifer.

It is outside the scope of this paper to discuss the epidemiology of the diseases caused by these organisms, but it may be permissible to draw attention to the importance of always distinguishing them clearly. As a single illustration it is sufficient to mention the suggestion, which their results led Bainbridge and O'Brien to put forward, that B. suipestifer and B. paratyphosus B have a different distribution in nature, the former occurring in the intestine of pigs and other animals, the latter in the human alimentary tract.

The Dysentery Group. Some authorities, such as Lentz, maintain that the dysentery organisms do not really belong to the Typhoid-Colon group. Practically, however, they must be included, since they have to be distinguished from other intestinal bacteria, which they closely resemble. In some respects they are the least satisfactory of the groups with which we have to deal, as in them we meet with a marked difference in grouping according as we take the cultural or the agglutination characters for our differentiating tests.

One of the varieties may be dismissed quite briefly, viz. the original B. dysenteriae of Shiga. This organism, which is non-motile, ferments glucose without production of gas, and after preliminary acidification turnus milk alkaline on or soon after the third day. It does not ferment mannite, dulcite, cane-sugar, lactose nor sorbite and does not produce indol. A somewhat similar organism has been described by Morgan, but it is motile and produces indol, and the agglutination reactions of the Shiga bacillus are characteristic.

It is when we come to the mannite-fermenting types of dysentery bacilli that difficulties arise. Some doubt has been expressed as to their claim to be considered genuine dysentery bacilli at all, owing to an alleged milder clinical course of the disease with which they are associated, but they frequently produce extremely severe or fatal dysentery, and a doubt of this kind is unjustified. An individual position is taken amongst them by the bacillus of Strong. This organism is unlike the rest in fermenting dulcite and cane-sugar, and in producing clot in milk, and its agglutination reactions also separate it from the others. As it has apparently been only once found in association with dysentery it is of subordinate importance, but we may notice that organisms not very dissimilar culturally and agglutinated by "Strong" serum have been found by Morgan to occur in the stools of typhoid convalescents.

The representative organism of the mannite-fermenting type is Flexner's B. dysenteriae, and it is the best known member of a group which possesses the following general characters. They are non-motile, ferment glucose and mannite without production of gas, turn milk alkaline after preliminary acidification, produce indol, and do not ferment lactose, dulcite or cane-sugar and do not liquefy gelatin. These group-characters are possessed by most of the mannite-fermenting dysentery organisms, though variations occur in the readiness with which they produce alkali in milk and form indol. Whether all intestinal organisms with these characters are potential producers of dysentery cannot be definitely stated as yet. It seems improbable, since they occur not infrequently in individuals with no history of dysentery, but sufficient evidence on the point is lacking. It is apparently questionable whether agglutinability by Shiga, Strong, Flexner or even by Y serum is a necessary property of all the pathogenic members of the group.

Flexner's organism ferments maltose and dextrin, and does not ferment sorbite, but other members of the sub-group show differences in one or more of these characters. This has recently been clearly shown by Morgan, who examined the behaviour toward eighteen carbohydrates of over 50 strains, and it has also been brought out by Tebbutt in his study of institutional dysentery in England, as well as by others. The best known of these, the Y bacillus of Hiss and Russell, does not ferment maltose in its original state, and therefore this sugar has been used as a basis of subclassification. We know, however, from Hiss's own work that the Y organism readily acquires the power of fermenting maltose, and there are other grounds for doubting the stability of the character. Bacillus Y resembles B. Flexner much more closely than do some other undeniable dysentery strains, e. g. some of the El Tor organisms of Ruffer and Willmore, and a primary subdivision on a maltose basis, even if the character were less variable than we know it to be, would separate Y and Flexner, while bringing together other organisms much less closely alike. It would appear to be more satisfactory to adopt a subdivision according to the more stable sorbite character as suggested by Tebbutt, and to redivide the subgroups so formed into those which do and those which do not ferment dextrin. This gives us four groups, each of which contains some dysentery organisms. It brings the Y bacillus into the same subgroup with the true Flexner, from which it can if desired be distinguished by the maltose test.

By no system of classification, however, can we bring the cultural and serological reactions into harmony. It appears that motile bacilli with claims on cultural grounds at admission to the group are definitely not agglutinated by dysentery sera, but otherwise the results are quite discordant. All observers are agreed that absorption methods are unsatisfactory for these organisms, and in every subgroup there occur some strains which are agglutinable by Flexner serum, and some which are not. We must recognise the fact that the biochemical and serum reactions are not at one in this group. Of course there is really no a priori ground for expecting them to agree, since variation of one character is not necessarily accompanied by variation in another. Between the two conflicting methods of classification we have no conclusive reason for preferring the one to the other, and we must wait for further experience on the subject. The impression one gains is that the dysentery organisms as a group are in a very unstable condition, and have not acquired fixed characters such as we find in other bacilli of the Typhoid-Colon class. A thorough examination of representative members from the mutation or variation point of view is much to be desired.

The details which have been given in the proceeding pages of this section enable us accurately and easily to subdivide such of the organisms of the Typhoid-Colon group as do not ferment lactose and do not liquefy gelatin into a number of distinct subgroups. The members of each subgroup resemble each other more or less closely, but they admit of a further subdivision by methods which are in most cases readily practicable. The resulting classification is summarised briefly in the following Table.

Table of organisms which neither ferment lactose nor liquefy gelatin.

I. **Late lactose-fermenters (p. 155 and p. 157).**
II. **Certain groups of no known pathogenic importance (p. 158).**
III. **Typhoid Group (p. 158).**
IV. **Paratyphoid-Enteritis Group.**
  1. **Atypical members.**
    a. **Saccharose-fermenters (p. 159).**
    b. **Dulcite non-fermenters (p. 159).**
    c. **B. paratyphosus A (p. 159).**
    d. **Salicin-fermenters (p. 160).**
  2. **Typical members (p. 158, 160 ff.).**
    a. **B. enteritidis Gärtner distinguished from b. and c. by agglutination.**
    b. **B. paratyphosus B (p. 160) distinguished from c. by absorption.**
    c. **B. suipestifer.**
V. **Dysentery Group.**
  1. **Mannite non-fermenting (p. 161) (B. dysenteriae Shiga).**
  2. **Mannite fermenting.**
    a. **B. dysenteriae Strong (p. 161).**
    b. **Sorbite-fermenters (p. 162).**
      I. **Dextrin non-fermenting.**
      II. **Dextrin-fermenting.**
    c. **Sorbite non-fermenters (p. 162).**
      I. **Dextrin non-fermenting.**
      II. **Dextrin-fermenting.**
        α. **Maltose-fermenting (B. dysenteriae Flexner).**
        β. **Maltose non-fermenting (B. dysenteriae Y).**

III.

In conclusion there are one or two general considerations which I wish to bring forward. The amount of work, which is done in connection with the typhoid-colon group every year and in all parts of the world, is simply enormous. Even if we omit the routine-examinations of such material as water, the work devoted to the investigation of faeces alone in connection with enteritis-cases carriers, foodpoisonings, genuine or suspected, and so on, is immense. This represents a large expenditure of energy, and unfortunately much of it does less than it might to advance our knowledge of bacteriology and the epidemiology of intestinal disease. Thereis a waste of energy in two directions. On the one hand, much time and labour are in many cases expended on the elaboration of points which contribute little or nothing to establishing the identity of the organism studied, and on the other much that is considered by many to be essential to a complete description is too often omitted.

The early bacteriologists for want of other diagnostic tests were compelled to lay stress on the appearance of colonies on the ordinary media, their vine-leaf shape, prominence, moistness, translucency and so on. Such points as these are of almost no value as differential tests. Even a blue colony on Conradi-Drigalski is of only elementary differential value, and the appearance of such colonies, their exact shade of blueness etc., does not take the distinction beyond the primary division into lactose-fermenters and non lactose-fermenters. But we still quite frequently find these details set out with laborious minuteness as if they contributed essential information in the distinction of the group-members from one another. Lengthy descriptions of this kind do not repay they trouble they demand, and will no doubt gradually disappear in time; but we are threatened with another cause of waste of energy in the multiplication of tests. The introduction of new tests is most desirable, for by them we may hope to deepen our knowledge, but when a test has received an extensive trial and has contributed nothing fresh to our knowledge, it should be dropped again. There are certain complicated media for example in frequent use for the growth of typhoid bacilli, which might very well lapse into disuse.

On the other hand I would urge a more general recognition of the value of the carbohydrate media. These tests supply most valuable help in the differentiation of the typhoid-like bacteria from one another. This is true of all the subgroups, but they are especially important in the case of the Gärtner-paratyphoid group, where we may meet so many organisms closely resembling the genuine pathogenic bacilli. Yet we find bacteriologists, even of great and deserved repute classifying organisms, whose dulcite or saccharose reactions have not been studied, as identical in fermentation activity with Paratyphoid B. Possibly they look on these points as of minor importance, but the want of information of this kind greatly reduces the value to others of the work done, and handicaps them in subsequent investigations on similar lines.

This is an exellent illustration of what really constitutes at present a great part of the difficulty which these organisms present, viz, the lack of uniformity in the methods by which they are studied. Bacteriologists in different countries and in different parts of the same country rely on different criteria in establishing a diagnosis, and as a consequence neglect the tests in which they happen to place less faith. To some extent this is inevitable, but it has the result of making the information collected by the followers of one set of criteria inadequate and therefore largely useless to those who adopt another set. Now taken altogether, the number of tests, employed by the leading bacteriologists who have devoted much time to the study of these intestinal bacilli, is not very large; and it should be possible for all workers to adopt as a basis a uniform standard of minimum requirements. This standard would not be exhaustive in the sense of including all known tests, and individual workers would naturally add other tests, either old or new, to which they attached importance. It need have nothing dogmatic about it, and would not commit those who adopted it to a belief in the validity of all the tests it included; and it would be subject to revision from time to time. But it would be comprehensive enough to cover all the most important tests an which bacteriologists of different schools are accustomed to rely. It would therefore ensure that work done in one part of the world by one school would be available to other schools elsewhere, and we should avoid the very real present difficulty, that the work of any one school is of full value only to that school and to no other. Something of this kind seems very much to be desired. Naturally it could be drawn up only by an association, at which the different methods of examination where adequately representated. If this Section of the International Congress of Hygiene and Demography were to appoint a representative Committee to draft recommendations of the nature indicated, it would take a step which it is well qualified to take, and one which would be of great value to all bacteriologists.

Résumé.

It is scarcely possible to summarise this paper briefly, but its scope may be indicated by the following.

Mutation or variation as it occurs in members of the typhoid-colon group is discussed in some detail, and it is pointed out that, while many members of the group are capable of such alteration towards one or more carbohydrates, this does not occur haphazard but in certain directions which are definite for each organism. An organism produces with greater readiness variants towards some carbohydrates than to others, and the more disposed an organism is to produce a particular variant, the more readily does it take the new character permanently. Variability is one of the characteristics of bacteria, and affects most of their known properties, and the fact that variation occurs towards carbohydrates does not invalidate their use as aids to differentiation. The carbohydrate media are the most elastic, most practical and most satisfactory means at present available of distinguishing the members of this group.

The primary division being made into those which do and those which do not ferment lactose, the non-lactose fermenters, which include the important pathogenic members, are considered in detail. The three prominent groups of typhoid, paratyphoid Gärtner, and dysentery are taken separately, and their characters and those of the organisms most closely resembling them are set out. Finally a plea is put forward for greater uniformity and if possible some standardisation of the methods employed in their study.

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ON THE INFLUENCE OF THE METAMORPHOSIS OF *MUSCA DOMESTICA* UPON BACTERIA ADMINISTERED IN THE LARVAL STAGE.

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A considerable amount of experimental work has been carried out by several workers to demonstrate that certain species of flies may infect food for varying periods of time after the fly is itself experimentally infected.

Till recently, the question as to whether pathogenic bacteria might be recovered from the interior of young flies, the larval stage of which had been contaminated with these bacteria, has not received much attention. During the past two years several investigators have attacked this question, but unfortunately their results are not strictly comparable owing to their having experimented with different species of flies and different organisms.

Bacot (1911) showed that if the larvae of *Musca domestica* be infected with *B. pyocyaneus* the resulting pupae and imagines undoubtedly remain infected with the bacillus.

Ledingham (1911) confirmed Bacot's results with *B. pyocyaneus* and then infected larvae with *B. typhosus*, but failed to recover the bacillus either in the grown larvae, pupae, or imagines. From the larvae were isolated other bacilli which had contaminated the ova and which he regarded as well adapted to the interior of the larvae, pupae, and imagines of *M. domestica*. Both the pupae and imagines were sometimes sterile but more often contained some of these persistent organisms, the most common being that described as "*Bac. A*" (Ledingham). After sterilization of the ova with lysol, however, and subsequent feeding of the young larvae with *B. typhosus*, this organism was recovered from the full-grown larvae and from the one pupa that he obtained.

Graham-Smith (1911) showed that if the larvae of the blow-fly (*Calliphora erythrocephala*) be infected with *B. anthracis* these bacilli may in a large proportion of cases be recovered from the young flies, but on the contrary he failed altogether to recover *B. typhosus*, *B. enteritidis*, *B. prodigiosus* or *V. cholerae* under the same conditions. He admits that "these experiments, however, afford no information as to the extent to which house-flies (*M. domestica*) bred from larvae fed on naturally infected excreta and similar materials are apt themselves to be infected."

Lucius Nicholls (1912) working independently in the Windward Isles has just recently published some interesting results. Using *Sarcophagula* he found that the number of organisms in the interior of pupae progressively diminished from the commencement of pupation to such an extent that if the number of bacteria in the grown larva be represented as 100, there was 0·4 left in the four-days old pupa. The absolute number of bacteria in the pupae of this fly, under more or less natural conditions, was less than 20 in seven pupae out of twelve.

Further, on breeding larvae of *Sarcophagula* in faeces infected with *B. typhosus*, *B. prodigiosus*, *Staphylococcus pyogenes aureus*, and a lactose-fermenting organism respectively, he found that these bacteria rapidly disappeared from the larvae if they were removed from their infected surroundings. From pupae developed from the larvae none of the bacteria were recovered.

Some pupae were watched and when about to hatch out, the imagines were seized, extracted with sterile forceps, and plated out. Of 12 imagines thus examined 2 were sterile and 10 gave only one or two colonies. A further batch of 12 imagines were allowed to emerge, placed immediately in a sterile vessel and kept for two days without food, after which every one was found to be sterile.

Of 12 imagines of another species (*Sarcophaga*), similarly treated, 10 were sterile.

Nicholls concludes that a freshly hatched fly may be considered as probably sterile and there is not much likelihood of its acquiring these organisms by contact with the material in which it bred.

The experiments which I will now detail were undertaken in order to confirm and elaborate certain points arising out of Ledingham's investigations summarized above. With regard to technique, Mr Bacot kindly supplied me with ova of *M. domestica* which were placed upon agar slopes to which a little fresh human blood was added together with the organisms with which the young larvae were to be infected. Sometimes the ova were sterilized by washing in 3 °/₀ lysol for two or three minutes. Larvae when full-grown were placed on sterile sand to pupate, and the pupae were stored in sterile test tubes till they were examined or till imagines emerged.

The method of examining the pupae was that described by Ledingham (1911), the blunt end being seared with a hot iron, a capillary pipette inserted and the contents sucked out and plated on MacConkey's neutral red lactose agar.

The imagines were examined either as soon as they were first noticed or after feeding on sterile cane sugar solution. The method used was to wash them separately in 2 °/₀ lysol for seven to ten minutes, then in two or three successive tubes of broth, after which each was crushed in another small amount of broth and the latter plated out on several MacConkey lactose plates, so that the whole of each imago was bacteriologically examined. The broths used to wash the fly were also incubated in order to detect bacteria on the external surface, and sub-cultures were made from the last broth in which the fly was washed before being crushed and plated.

The ova, larvae, pupae and imagines were kept throughout at a temperature of 25° C

With regard to the bacteria met with and employed in this research, a few comments are necessary. The principal pathogenic organism on which larvae were fed was one of the mannite types of the dysentery bacillus, viz. the *Bac.* "*Y*" of Hiss and Russell.

The organism referred to as "*Bac. A*" was described by Ledingham and corresponds in its fermentation reactions with Morgan's *No. IV B*, a non-lactose fermenter from the faeces of children. "*Pseudo No. 1*" is a provisional name for a motile non-liquefying bacillus giving acid and gas in glucose media and alkalinity in litmus milk, like Morgan's *No. 1*. It differs from the latter (1) in fermenting adonite with gas production, (2) in not fermenting galactose, (3) in producing more marked alkalinity in litmus milk, (4) in producing acidity in saccharose in five days, (5) in a slower production of indol. In spite of these differences it is apparently more closely allied to Morgan's *No. 1* than to any other non-lactose fermenter. I have twice isolated it from human faeces as well as from flies and it is worth noting, in view of the fact that Morgan's *No. 1* has been said to be rather common in flies, that these two bacilli would not have been differentiated in all probability unless galactose or adonite media had been used.

Series I.

Technique.	*Bacteria found.*
Ova, not sterilized, placed on blood agar.	Contaminating the eggshells and young larvae were: Types of *B. coli*, Gelatin-liquefying cocci, "*Bac. A*" (Ledingham).
Two larvae, 2 days old, washed in lysol and plated out.	*Pseudo No. 1* bacilli found in each.
All larvae washed in lysol when 2 days old, and *Bac.* "*Y*" (Hiss and Russell) mixed with blood on fresh agar slopes on which the larvae were placed.	
One larva, 6 days old washed in lysol, and plated out.	"*Bac. A.*"
Remaining larvae placed on sterile sand.	
7 pupae examined. 1 kept and fly emerged.	6 pupae sterile. 1 pupa gave about 30 colonies, a mixture of *Pseudo No. 1* and "*Bac. A.*"

Summary of Series I. Of the numerous types of bacteria found in association with the ova and young larvae, only those called *Pseudo No. 1* and "*Bac. A.*" persisted, although 6 pupae out of 7 had become sterile. The mannite type of dysentery bacilli administered was not found either in larvae or pupae.

Series II.

Technique.	*Bacteria found.*
Ova, not sterilized, placed on blood agar.	Associated with 1-day old larvae Gelatin-liquefying cocci, *Pseudo No. 1* bacillus.
4-days old larvae washed with 0·25 % lysol (40 minutes) then with broth. Fed with blood and *Bac.* "*Y*" on agar for 5 days then placed on sand to pupate.	
3 pupae obtained.	1 pupa sterile. 1 pupa: *Pseudo No. 1* bacillus, Lactose fermenting gram-negative bacilli. 1 pupa: Gram positive cocci.

Summary of Series II. As before *Bac.* "*Y*" was not recovered from pupae. *Pseudo No. 1* bacillus again persisted in one pupa. One pupa sterile out of 3 examined.

Series III.

Technique.	*Bacteria found.*
Several ova incubated in broth and then plated out.	Lactose fermenters. "*Bac. A.*"
Remaining ova washed with 0·5 % carbolic for 3 minutes and placed on agar. Sterilization not effected for growth appeared in association with the young larvae.	
5 days old larvae washed with 0·25 % lysol (30 minutes) then with broth. Fed for 4 days with blood and *Bac.* "*Y*," then placed on sand to pupate.	
7 pupae examined.	4 pupae sterile. 2 pupae contained both *Bac. A* and lactose fermenters. 1 pupa "*Bac. A*" and a dulcite fermenter.
One fly found about 1 hour after emergence and allowed to walk over a MacConkey plate, on which a growth took place.	"*Bac. A.*" Gram positive cocci.

Summary of Series III. "*Bac. A*" persisted as in Series I. "*Bac. Y*" not recovered from pupae or fly. Four pupae sterile out of 7.

Series IV.

Technique.	*Bacteria found.*
Ova washed with 3 % lysol for 2–3 minutes, then with normal saline. Larvae hatched and remained sterile being fed with fresh blood. When 3 days old fed with "*Bac. Y*" and blood.	
When 9 days old and full grown placed on sand to pupate.	
4 pupae examined.	3 pupae sterile. 1 pupa contained about 60 colonies, all non-lactose-fermenters, 4 of which were further examined and found to be *Bac. Y.* This pupa was 4–7 days old when examined.
1 pupa left and a normal imago emerged but was not examined.	

Summary of Series IV. Larvae hatched from sterilized ova were bred and afterwards fed in contact with a dysentery bacillus which was recovered from 1 pupa out of 4.

Series V.

Technique.	*Bacteria found.*
Ova washed with 3 $^0/_0$ lysol for 2 minutes then with normal saline. Larvae sterile as before and fed with blood and *Bac. Y* from the second day till mature. Then placed on sand. 5 pupae examined.	3 pupae sterile. 1 pupa gave 1 colony of *Bac. Y.* 1 pupa gave 2 colonies of *Bac. Y.*
1 pupa washed with 3 $^0/_0$ lysol for 7 minutes and placed on a sterile plate. An abnormal imago emerged and lived for 36 hours and was then crushed and plated.	Fly sterile.
1 pupa treated as above but no imago appeared. After about 10 days the contents which were semi-solid were plated.	1 pupa about 10 days old contained 50 colonies (*Bac. Y*). (Possibly this pupa was dead and the organisms had afterwards multiplied.)

Summary of Series V. Dysentery bacilli found to persist though in very small numbers in 2 pupae out of 5. 1 fly found to be sterile.

Series VI.

A. One batch of eggs was washed with lysol as before and the sterile larvae were fed with *Bac. Y* and fresh blood.

1 pupa was obtained and found to be sterile.

B. A second batch of eggs was treated with lysol but the larvae were contaminated and were allowed to mature without administering other bacteria.

4 pupae were obtained, all sterile.

Summary of Series VI. Batch B shows that some bacteria will resist treatment with lysol but yet fail to persist in the metamorphosis from larva to pupa.

Series VII.

Technique.	*Bacteria found.*
A. One batch of ova washed with lysol as before and the sterile larvae fed with *Bac. A* (Ledingham).	
1 pupa examined.	About 170 colonies of *Bac. A* (identified).
B. Another batch treated in the same way.	
1 pupa examined (4 days old).	About 500 colonies of *Bac. A* (identified).
1 pupa left and the emerging imago washed in 2 $^0/_0$ lysol for 7 mins. and then in successive broths, then crushed and plated out.	Fly sterile.
Another imago fed for 3 days on sterile sugar solutions. This fly died and was plated out.	Fly sterile.
Another imago was plated out a few hours after emergence after washing with lysol and broths.	Fly gave very numerous colonies, not further identified as *Bac. A* other than that they gave the peculiar sour odour of *Bac. A* and were non-lactose-fermenters.

Summary of Series VII. "*Bac. A*," found naturally associated with flies, was recovered from 2 pupae examined in large numbers, and almost certainly from 1 fly out of 3.

Series VIII.

Ova were sterilized as before and fed with blood and *Bac. Y.* The pupae obtained were placed in sterile test tubes and the flies examined as soon after emergence as possible. If alive they were chloroformed, then washed in 2 $^0/_0$ lysol for about 5 minutes and finally in successive broths. The fly was then crushed in broth and plated out on several MacConkey plates.

Fly 1. Broths clear and plates sterile.

Fly 2. Broths clear and plates sterile.

Fly 3. Broths clear. Three plates used, each showed a number of pale colonies, one of which was taken and identified as *Bac. Y.*

Fly 4. Broths clear. One plate showed numerous pale colonies and a few sarcina-like colonies.

Fly 5. Broths clear and plates sterile.

Fly 6. This did not develop its wings and died soon after emergence. The broths used for washing became turbid and the plates were thickly covered with pale colonies, one of which was identified as *Bac. Y.*

Summary of Series VIII. Two flies of normal appearance were found to contain a dysentery bacillus which had been administered to larvae reared from sterilized ova.

Series IX.

Ova treated with lysol as before and some larvae fed with *Bac. A* and some with *B. pseudo No. 1.*

I am unable to give the results separately, but of 8 flies examined 6 were sterile whilst 2 gave numerous colonies, the broth washings being clear.

Series X.

A. A batch of larvae from sterilized eggs were fed with *B. typhosus.* The one fly obtained proved to be sterile.

B. From a similar batch of larvae hatched from sterile ova and fed with *B. typhosus* 15 flies were obtained, but *B. typhosus* was not recovered in any one of them.

C. In a further batch 6 pupae and 16 flies were examined, all negative for the *B. typhosus.*

Summary of Series X. *B. typhosus* failed to persist in the metamorphosis from larva to imago in every case examined.

It must be remembered that the conditions in many of these experiments have been highly artificial. *Musca domestica* does not specially seek human excrement for the purpose of depositing its eggs, and in human excrement only are the bacilli of dysentery and typhoid fever likely to be found in any number. Numerous other types of bacilli are associated in nearly all cases with these pathogenic bacteria, and it has been shown by several workers that in the case of the *B. typhosus* and, in some of the above experiments, in the case of a dysentery bacillus, that where larvae are bred in association with a mixed flora, that is in more or less natural conditions, these pathogenic bacteria can rarely be recovered from the larval interior and certainly not from the pupae. The possibility then of an emerging fly being already infected with either of these bacilli seems to be very remote. Lucius Nicholls working with other species found that emerging flies were under natural conditions either sterile or contained one or two colonies only.

Under the artificial conditions where the ova were sterilized and larvae only allowed to come into contact with the organism administered it was found in the case of the dysentery bacillus used that a large number of pupae and imagines were sterile, but that in some cases a few bacilli persisted through the metamorphosis. On the other hand, *B. typhosus* was never recovered in either pupae or imagines though it is worthy of mention that Ledingham recovered a few bacilli from one pupa under similar experimental conditions.

The general impression that one gathers from the work already published and from these experiments is that during the metamorphosis a marked inhibition is exhibited towards certain bacteria ingested in the larval stage. It does not seem to take effect upon all bacteria; for example, Bacot found *B. pyocyaneus* appearing very constantly in the pupae and imagines.

The factors affecting bacteria in a larva commencing to pupate may vary according as to whether the organisms are in pure or mixed culture. In the latter case it is possible that one variety of organism may injuriously affect the growth of the other and this inhibition will then be superadded to the definite bactericidal action of metamorphosis itself, however produced; on the other hand, if only one species of organism is present, a symbiotic effect cannot take place. An attempt was made to extract bactericidal substances from larvae. A large number of larvae were crushed in saline in a mortar and the emulsion passed through a Berkefeld filter. An emulsion of *Bac. Y* (of dysentery) was then added to different dilutions of the larval extract, but the extract was not found to be so prejudicial to this organism as normal saline itself.

Ledingham concluded that certain bacteria found commonly in flies had a special adaptability to the metamorphosis and were able to persist in the insects throughout their metamorphosis. Not even these types, however, are always able to withstand the inhibitory effect though they do so far more successfully than do such bacteria as *B. typhosus* and *Bac. Y* of dysentery. I found that the latter bacillus does not grow so well at the temperature of a fly (room temperature 60°–75° F.) as does *Bac. A* of flies, so that temperature may play some part in increasing the relative number of this latter bacillus. Whether the normal flora of the house-fly exerts a direct inhibitory effect on the growth of pathogenic bacilli was not thoroughly investigated, but it was found in one series of experiments that where *Bac. Y* and *Bac. A* (of flies) were inoculated in about equal numbers into broth and incubated at 37° C., in 24 hours *Bac. Y* had diminished to 36 %, and in 48 hours to 25 %. Possibly the truth lies in a combination of these explanations, viz. that the metamorphosis of the fly exerts a very marked inhibitory effect on certain bacteria as, for example, the pathogenic species above mentioned, and that where other bacteria are present, which are better adapted both to their environment and temperature, this prejudicial effect is still more marked, accounting for the result that by the time the pupa is formed none of these pathogenic bacteria can be recovered.

SUMMARY AND CONCLUSIONS.

(1) Pathogenic organisms such as *B. dysenteriae* (type "*Y*") cannot be recovered from pupae or imagines reared from larvae to which these organisms have been administered.

(2) When the larvae have been bred from disinfected ova and are subsequently fed on *B. dysenteriae* (type "*Y*"), this organism may be successfully recovered from the pupae and imagines in a small proportion of cases.

(3) Under similar conditions *B. typhosus* was not recovered in a single case from pupae or imagines.

(4) In those cases in which *B. dysenteriae* (*Y*) was successfully recovered from pupae, the colonies on the plate were invariably fewer than those obtained from pupae and imagines after administration to the larvae of more adaptable organisms such as "*Bac. A*" (Ledingham).

(5) When organisms such as "*Bac. A*" were administered to larvae bred from disinfected ova, or non-disinfected ova contaminated with this organism, it was in many cases possible to recover the organism from pupae and imagines.

(6) In no series of pupae examined after administration to the larvae of either *B. dysenteriae* or "*Bac. A*" was it possible to recover the organisms in every instance. A certain proportion of pupae in both cases proved sterile, so that the process of metamorphosis is undoubtedly accompanied by a considerable destruction of the bacteria present in the larval stage.

(7) The temperature at which the larvae develop (19°–25° C.) has probably an important bearing on the survival of pathogenic organisms such as "*Bac. Y*" of dysentery, administered in association with organisms such as "*Bac. A*," in view of the fact that the latter grows far more luxuriantly at this temperature. Even when grown in broth at 37° C. (the optimum temperature for *Bac. Y*) together with "*Bac. A*," the "*Bac. Y*" was found to form after two days only one quarter of the total number of bacteria present in the mixed growth.

(8) There was no evidence that the larval juices contained substances bactericidal for *Bac. Y*. The bacilli died more rapidly in normal saline solution.

(9) The possibility of flies becoming infected from the presence of pathogenic organisms in the breeding ground of the larvae may be considered as very remote.

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